

Sonication ChIP Kit

RK20258



Instruction Manual

Version:N19E20V1.1

Contents

1. Introduction	1
2. Components	1
3. Storage and Shipping Conditions	1
4. Additional Required Materials	2
5. Workflow	3
6. Precautions	4
7. Protocol	6
8. Appendix	13

1. Introduction

Sonication ChIP Kit (RK20258) provides a protocol and reagents for running ChIP assays including: cell lysis and chromatin extraction, chromatin shearing and DNA fragment analysis, immunoprecipitation and DNA purification. Obtained ChIP DNA using the kit can be analyzed by qPCR or NGS after DNA library preparation. The kit has been validated for ChIP assays with mammalian samples.

2. Components

Box		Component (with tube color)	Cat.No.	Size
				24 RXN
Box A		Glycine Solution (10X)	RM20700	10 mL
		Cell Swelling Buffer (10X)	RM20701	5.3 mL
		ChIP Sonication Buffer (10X)	RM20702	4.3 mL
		ChIP Low Salt Wash Buffer (10X)	RM20703	5.7 mL
		ChIP High Salt Wash Buffer (5X)	RM20704	10 mL
		LiCl Buffer (5X)	RM20705	5.3 mL
		TE Buffer (100X)	RM20706	580 µL
		ChIP Elution Buffer	RM20707	8 mL
		5 M NaCl	RM20708	700 µL
Box B		1 M DTT	RM20164	100 µL
		Proteinase K (20 mg/mL)	RM20713	210 µL
		RNase A (10 mg/mL)	RM20709	210 µL
		Human RPL30 Exon2 Primers (5 µM)	RM20710	160 µL
		ChIP-Grade Histone H3 Antibody (1 mg/mL) *	RM20711	20 µL
		IgG (1 mg/mL)*	RM20712	20 µL

Notes:

- (1) Antibodies (H3 and IgG) are provided in limited quantities for testing. Additional amounts must be purchased separately (see Section 4.3).
- (2) Avoid >10 freeze-thaw cycles for control antibodies.
- (3) Store reagents under specified conditions to maintain performance.
- (4) Dissolve DTT by incubating at 25°C for 10–15 min or at room temperature for 15–20 min. Vortex 20x if precipitates remain.

3. Storage and Shipping Conditions

Shipping: Box A is shipped with ice packs; Box B is shipped on dry ice.

Storage: Store Box A at 4°C; Box B at -20°C. All components remain stable for 1 year under proper storage.

4. Additional Required Materials

4.1. IP Reagents/Consumables

- High-quality Formaldehyde (16%)
- PBS Buffer (1X) (ABclonal, RM00012)
- Protease Inhibitor Cocktail (100X)
- ChIP-grade Protein A/G Magnetic Beads
- DNA purification kit: AFTSpin Multifunction DNA Purification Kit (ABclonal, RK30100)
- Absolute ethanol
- Nuclease-free Water (ABclonal, RK20102)
- Magnetic Separation Device

4.2. qPCR and NGS Library Prep Reagents

- qPCR mix: 2X Universal SYBR Green Fast qPCR Mix (ABclonal, RK21203)
- DNA library prep kits: Rapid Plus DNA Lib Prep Kit for Illumina V2 (ABclonal, RK20255); Scale ssDNA-seq Lib Prep Kit for Illumina V2 (ABclonal, RK20228)

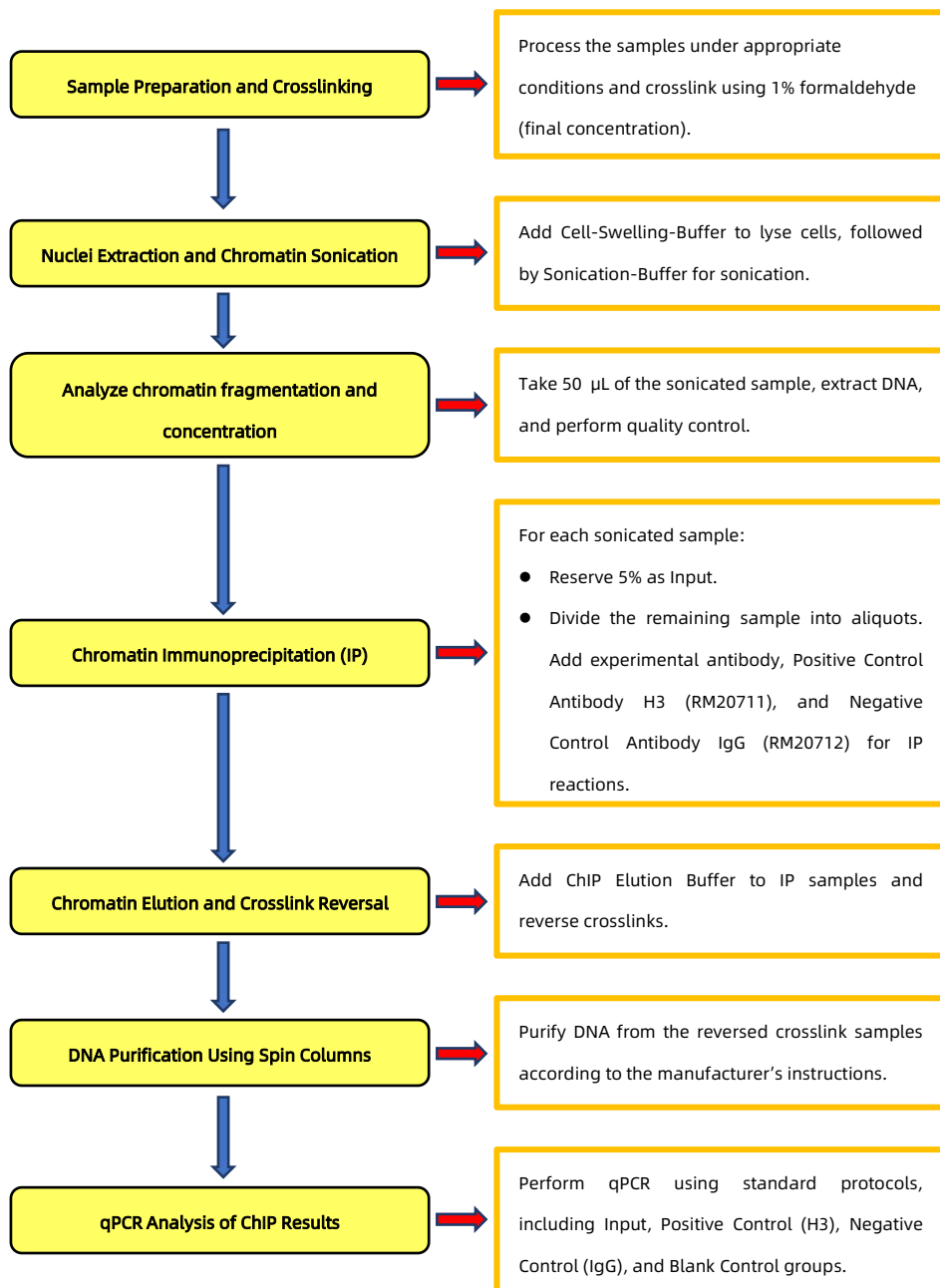
4.3. Antibodies

- Target-specific ChIP-grade antibody (refer to ABclonal website)
- Positive control: ChIP-Grade Histone H3 Antibody (1 mg/mL) (ABclonal, A17562)
- Negative control: Rabbit Control IgG (ABclonal, AC005)

4.4. DNA Quantification

- Qubit Fluorometer
- Qubit dsDNA HS Assay Kit (Thermo Fisher, Q32854) or ABQubit 1X dsDNA HS Assay Kit (Pre-Mixed) (ABclonal, RK30140)

5. Workflow



6. Precautions

6.1. General Guidelines

- Thaw all reagents completely before use. Briefly centrifuge to collect liquid at the bottom of the tube. Reagents can be thawed fully at room temperature.
- During use, keep Box A at room temperature and Box B on ice. After use, promptly return all components to their specified storage conditions.
- Dilute stock solution of Cell Swelling Buffer, Sonication Buffer, ChIP Low Salt Wash Buffer, ChIP High Salt Wash Buffer, LiCl Buffer, TE Buffer with nuclease-free water to 1X working concentration, and store at 4°C before using.

Note: 1X buffers can be stored at -20°C for up to 6 months or at 4°C for up to 2 months.

For 24 RXN Kit:

Box	Component	Cat.No.	Stock Solution	Add ddH ₂ O	Total Volume
Box A	 Cell Swelling Buffer (10X)	RM20701	5.3 mL	47.7 mL	53 mL
	 ChIP Sonication Buffer (10X)	RM20702	4.3 mL	38.7 mL	43 mL
	 ChIP Low Salt Wash Buffer (10X)	RM20703	5.7 mL	51.3 mL	57 mL
	 ChIP High Salt Wash Buffer (5X)	RM20704	10 mL	40 mL	50 mL
	 LiCl Buffer (5X)	RM20705	5.3 mL	21.2 mL	26.5 mL
	 TE Buffer (100X)	RM20706	580 µL	57.4 mL	58 mL

6.2. Sample Requirements

- Tissue Samples:

For optimal ChIP results, use 25 mg of tissue for each immunoprecipitation to be performed. When harvesting tissue, remove unwanted material from the sample, such as fat and necrotic material. Crosslinking is recommended immediately after sample collection. In practical, it is recommended to prepare about 100 to 150 mg tissue samples for one sample group for subsequent immunoprecipitation reactions and technical replicates. For example, chromatin should be enough for immunoprecipitation with negative control (IgG), positive control H3 antibody and target protein antibody, separately.

- Cell Samples:

For optimal ChIP results, use approximately 4×10^6 cells for each immunoprecipitation to be performed. In practical, it is recommended to prepare about 1×10^7 to 2×10^7 cells (For 293T cells, this typically corresponds to 2-3 10 cm culture dishes with cells grown to $\geq 90\%$ confluency) for one sample group for subsequent immunoprecipitation reactions and technical replicates. For example, chromatin should be enough for immunoprecipitation with negative control (IgG), positive control H3 antibody and target protein antibody, separately.

6.3. Control Antibodies and ChIP-qPCR Primers

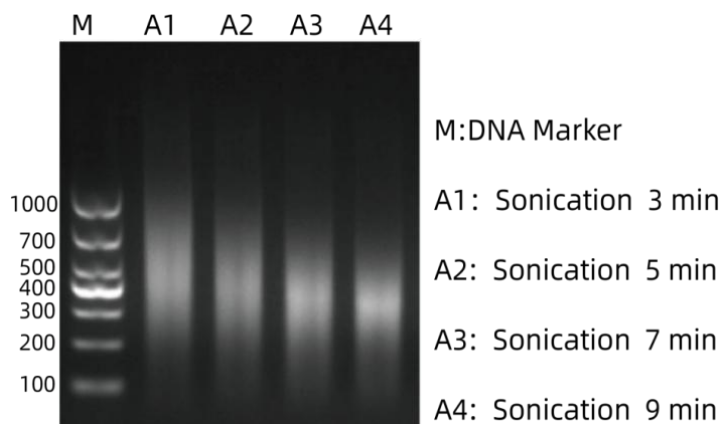
- The kit provides negative control antibody, Rabbit IgG antibody, positive control antibody: ChIP-grade Histone H3 antibody, and Human RPL30 Exon 2 primers.

- Histone H3 binds to most DNA sequences in the genome, including the RPL30 locus, while the IgG antibody does not bind DNA. qPCR analysis can be used to evaluate gene enrichment and confirm experimental success.
- RPL30 primers are designed for exon 2 of the human RPL30 gene. Sequences are listed below. For mouse or other species, prepare species-specific primers.

Primer	Sequence (5' to 3')	Base pairs (bp)
Human RPL30 Exon2 Primer-F (100 μM)	CCTCACTCACCGTCTCTTTG	21
Human RPL30 Exon2 Primer-R (100 μM)	TTGCGGGTTGCCATTGT	18

6.4. Sonication Optimization

6.4.1. We suggest to preparing extra cell samples for testing sonication conditions, checking chromatin shearing efficiency and chromatin concentration. The sonicator conditions should be tested in advance, including sonication power, cycles and duration time for each cycle. Sonication conditions could be determined by checking the electrophoresis results of purified chromatin DNA after different sonication cycles. If desired, 3-5 additional chromatin samples should be processed for optimization of chromatin shearing by sonicator. **The ideal range of sheared DNA fragments is expected as 60% to 90% of the chromatin fragments less than 1 kb.**



Using a contact-type ultrasonic homogenizer (Scientz-IID), chromatin from 1×10^7 293T cells was fragmented under the following conditions: 50 W power, with 1 s on/1 s off cycles. According to the electrophoresis results, DNA fragment sizes after 3 minutes of sonication met the requirements for qPCR detection, while those after 9 minutes of sonication were suitable for NGS library construction.

6.4.2. Guidelines for Different Ultrasonic Homogenizers

Ultrasonic Homogenizer Type	Recommended Tube	Volume of samples (μL)
Contact Probe Ultrasonic Homogenizer	2.0 mL tube	500-1000
Non-Contact Ultrasonic Homogenizer	1.5 mL tube	300-500
	0.2 mL tube	50-150

Contact Probe Ultrasonic Homogenizer: It is recommended to use a 3 mm or 2 mm diameter microprobe with an amplitude setting below 50% (optimization required). Employ a sonication cycle of 2 seconds on and 2 seconds off (users may adjust sonication duration, interval time, and cycle number according to their needs). Optimize sonication conditions and duration in advance. Subsequently, sonicate chromatin samples under the optimized parameters to obtain ideal fragmented chromatin. During sonication, keep samples on ice at all times. Avoid contact between the probe and the bottom/wall of the sonication tube. If foaming occurs, pause sonication and reposition the tube.

Non-Contact Ultrasonic Homogenizer: Refer to the instrument manual to set appropriate power (typically 50–100 W for samples $\leq 500 \mu\text{L}$), sonication cycles, and total runtime based on sample volume. Prior to each ChIP experiment, optimize sonication parameters for different sample types using time gradients (e.g., 1 min, 2 min, 3 min, 4 min, 5 min). Recommended settings: ≤ 10 seconds per sonication pulse, ≥ 10 seconds interval between pulses. A reference protocol: 10 seconds on, 20 seconds off, total runtime 12 minutes (for 1×10^7 cells).

Prolonging the cross-linking time or using excessively high cross-linking temperatures will significantly reduce chromatin fragmentation efficiency during sonication. Additionally, excessive sonication may damage antigenic epitopes on chromatin, leading to reduced antibody enrichment efficiency and subsequent issues such as weakened ChIP amplification signals. Therefore, we recommend using the minimal number of sonication cycles required to achieve ideal chromatin fragment sizes when processing samples. If extended sonication still fails to produce chromatin fragments within the desired size range, it is recommended to prepare new samples.

Note: Excessive sonication volume or high chromatin concentration can reduce fragmentation efficiency. To improve fragmentation efficiency, consider dividing one sample into multiple tubes for sonication. These tubes can later be combined into a single tube for subsequent experimental steps.

7. Protocol

7.1. Cell Culture Cross-linking and Sample Preparation

7.1.1. Sample Collection

The following procedure uses human cells as an example: Collect 1×10^7 cells in a 1.5 mL EP tube. For tissue sample processing, refer to Appendix.

7.1.2. Freshly prepare **Cell Fixation Solution** (Final formaldehyde concentration is 1%)

Reagent	Volume
1X PBS Buffer (PBS)	1 mL
16% Formaldehyde Solution	62.5 μL
Total Volume	~1 mL

Note: Prepare fresh and use immediately.

7.1.3. Add 1 mL of freshly prepared **Cell Fixation Solution** to the sample. Mix thoroughly by pipetting up and down. Incubate on ice for 15 min, gently shaking periodically.

7.1.4. Stop cross-linking by adding 100 μ L of Glycine Solution (10X) to the sample. Mix and incubate on ice for 5 min,, gently shaking periodically.

Note: Pre-warm Glycine Solution (10X) at 37°C before use.

7.1.5. Centrifuge cells at 1,200 x g for 5 min at 4°C, discard the supernatant.

7.1.6. Wash cells two times with 1 mL ice-cold 1X PBS Buffer (PBS).

7.1.7. Centrifuge cells at 1,200 x g for 5 min at 4°C, discard the supernatant.

Note: Cell pellets can be stored at -80°C for later use. To proceed, continue to Step 7.2.

7.2. Nuclei Preparation and Chromatin Fragmentation

Note: The reagent volumes listed below are for one sample. For multiple samples, multiply by N.

➤ Nuclei Preparation

7.2.1. Prepare **Cell Lysis Buffer** (Cell Swelling Buffer (1X)+PIC+DTT)

	Reagent	Volume
	Cell Swelling Buffer(1X)	2 mL
	100X PIC*	20 μ L
	1M DTT	2 μ L
	Total Volume	~2 mL

Notes:

(1) 100X PIC (Protease Inhibitor Cocktail) must be prepared by the user. Make sure it is completely thawed prior to use.

(2) Add PIC and DTT immediately before use.

(3) All operations must be performed on ice or under cold conditions.

7.2.2. Add 1 mL of ice-cold **Cell Lysis Buffer** (Cell Swelling Buffer (1X) + PIC + DTT) to resuspend the cells. Incubate at 4°C on a rotary shaker at the lowest speed for 10 min.

7.2.3. Centrifuge at 5,000 x g for 5 min at 4°C to pellet the nuclei. Remove supernatant.

7.2.4. Add 1 mL of ice-cold **Cell Lysis Buffer** (Cell Swelling Buffer (1X) + PIC + DTT) to resuspend the pellet. Incubate at 4°C on a rotary shaker at the lowest speed for 5 min.

7.2.5. Centrifuge at 5000 x g for 5 min at 4°C to pellet the nuclei.

➤ Chromatin Fragmentation

7.2.6. Prepare **ChIP Sonication Nuclear Lysis Buffer** (ChIP Sonication Buffer (1X)+PIC+DTT)

	Reagent	Volume
	ChIP Sonication Buffer(1X)	1 mL
	100X PIC*	10 μ L
	1M DTT	1 μ L
	Total Volume	~1 mL

Notes:

(1) 100X PIC must be prepared by the user. Make sure it is completely thawed prior to use.

(2) Add PIC and DTT immediately before use.

(3) All operations must be performed on ice or under cold conditions.

7.2.7. Add 1 mL of **ChIP Sonication Nuclear Lysis Buffer** (ChIP Sonication Buffer (1X)+PIC+DTT) to resuspend the pellet. Incubate at 4°C on a rotary shaker at the lowest speed for 20 min, and briefly centrifuge.

7.2.8. Transfer the sample to a 1.5 mL microcentrifuge tube suitable for sonication.

7.2.9. Fragment chromatin by sonication.

Example: Using a Scientz-IID contact sonicator, insert the probe halfway into the liquid (1.5 mL tube placed on ice).

Cell samples: 40 W, 1 s ON / 1 s OFF, 15 min.

Tissue samples: 65 W, 1 s ON / 1 s OFF, 15 min.

(Optimize parameters based on laboratory-specific instruments and sample types.)

7.2.10. After sonication, centrifuge at 12,000 × g for 10 minutes at 4°C.

7.2.11. Transfer the supernatant to a new tube. This is the cross-linked chromatin preparation, which can be used immediately for immunoprecipitation (see Step 7.4.2), or stored at -80 °C until further use. Remove 50 µL of the chromatin preparation for Analysis of Chromatin Fragmentation and Concentration.

7.3. Analysis of Chromatin Fragmentation and Concentration

7.3.1. To the 50 µL chromatin sample from step 7.2.11, add 100 µL of Nuclease-free Water to adjust the total volume to 150 µL. Then add 4.8 µL of 5 M NaCl and 2 µL of RNase A (10 mg/mL). Vortex to mix and incubate at 37°C for 30 min.

7.3.2. To each RNase A-digested sample, add 2 µL Proteinase K (20 mg/mL). Vortex to mix and incubate at 65 °C for at least 2 hr.

➤ **DNA Extraction (Using ABclonal AFTSpin Multifunction DNA Purification Kit [RK30100] as an Example):**

7.3.3. (Optional) Add an equal volume of isopropanol to the sample and mix by vortexing.

7.3.4. Add 750 µL Buffer DB to each tube at a 1:5 ratio (sample volume : buffer volume). Mix thoroughly. If the total volume exceeds 550 µL, process in batches.

7.3.5. Transfer the solution to a Spin Column and centrifuge at 12,000 × g for 1 min. Discard the flow-through and return the Spin Column to the collection tube.

7.3.6. Add 600 µL Buffer W1 (ensure ethanol has been added as specified) to the Spin Column. Let stand for 2 min, then centrifuge at 12,000 × g for 1 min. Discard the flow-through.

7.3.7. Repeat Step 7.3.6 once.

7.3.8. Return the Spin Column to the collection tube and centrifuge at 12,000 × g for 2 min.

- 7.3.9. Transfer the Spin Column to a clean 1.5 mL tube. Open the lid and air-dry at 20–25°C for 2 min to remove residual ethanol.
- 7.3.10. Add 50 µL Nuclease-free Water (pre-warmed to 65–70°C for higher efficiency) to the center of the membrane. Incubate at room temperature for 2 min, then centrifuge at 12,000 × g for 2 min. For higher DNA yield, reload the eluate onto the column and centrifuge for 1 min.
- 7.3.11. Discard the Spin Column. The eluate in the centrifuge tube is the purified DNA.
- 7.3.12. Remove a 10 µL sample and determine DNA fragment size by electrophoresis on a 1% agarose gel. Approximately 60 to 90% of total DNA fragments should be within the 200–700 bp size range.
- 7.3.13. Determine DNA concentration using a Qubit fluorometer. Ideally, DNA concentration should exceed 50 ng/µL.

7.4. Chromatin Immunoprecipitation

➤ Antibody-Target Protein Incubation

- 7.4.1. Prepare **ChIP Sonication Nuclear Lysis Buffer** (ChIP Sonication Buffer (1X)+PIC+DTT)

	Reagent	Volume
	ChIP Sonication Buffer(1X)	1 mL
	100X PIC*	10 µL
	1M DTT	1 µL
	Total Volume	~1 mL

Note: All operations must be performed on ice or under cold conditions.

- 7.4.2. Add an equal amount of fragmented chromatin sample (10–15 µg, from Step 7.2.11) to each IP reaction tube. Adjust the total volume to 500 µL using **ChIP Sonication Nuclear Lysis Buffer** (ChIP Sonication Buffer (1X)+PIC+DTT).
- 7.4.3. From the diluted chromatin in Step 7.4.2, transfer 25 µL to a new tube as a 5% Input control. Store at –20°C until further use.

Note: The Input sample will undergo reverse crosslinking alongside IP samples in Step 7.5.2.

- 7.4.4. To each target protein sample tube, add the corresponding antibody. For negative controls, add normal isotype IgG. For positive controls, add 5 µg of Histone H3 antibody. Adjust antibody amounts based on manufacturer recommendations (typically 2–5 µg per IP). Incubate at 4°C for ≥3 hr with rotation (avoid overnight incubation).

Note: In ChIP experiments, the antibody amount is critical. Excessive or insufficient quantities may adversely affect target enrichment.

➤ Protein A/G Magnetic Beads Pretreatment

- 7.4.5. Resuspend ChIP-Grade Protein A/G Magnetic Beads by gently vortexing. Transfer 30 µL beads per IP sample.

Note: Trim the pipette tip to facilitate bead aspiration.

- 7.4.6. Add 200 μ L 1X ChIP sonication buffer. Mix, place on a magnetic separation rack until the solution clears (1-2 min), and discard the supernatant.
- 7.4.7. (Optional) Add 200 μ L 3% BSA (prepared in TE buffer). Incubate at 4°C on a rotary shaker at the lowest speed for 1 hr. Wash beads twice with 200 μ L 1X ChIP sonication buffer.

➤ **Antibody-Target Protein Complex Incubation with Protein A/G Magnetic Beads**

- 7.4.8. Centrifuge the reaction mixture from Step 7.4.4 at 10,000 $\times$ g for 10 s. Transfer all the reaction mixture to pre-treated Protein A/G magnetic bead tubes. Seal tubes and incubate on a rotor at 4°C for 2 hr.
- 7.4.9. Place tubes on a magnetic separation rack. Wait 1 to 2 min for solution to clear and then carefully remove supernatant (retain temporarily for troubleshooting).
- 7.4.10. Add 200 μ L 1X ChIP Sonication Buffer, vortex, and discard supernatant.

➤ **IP Complex Washing**

- 7.4.11. Add 500 μ L of low salt wash (1X ChIP Low Salt Wash Buffer), remove the sample tube from the magnetic separation rack, incubate at 4°C for 5 min with rotation. Return the sample to the magnetic separation rack, wait 1-2 min for the solution to clear, and carefully remove the supernatant.
- 7.4.12. Add 1 mL of high salt wash (1X ChIP High Salt Wash Buffer), remove the sample tube from the magnetic separation rack, incubate at 4°C for 5 min with rotation. Return the sample to the magnetic separation rack, wait 1-2 min for the solution to clear, and carefully remove the supernatant.
- 7.4.13. If preliminary experiments indicate strong nonspecific antibody adsorption, add 1 mL of LiCl Wash Buffer (1X LiCl Buffer), remove the sample tube from the magnetic separation rack, incubate at 4°C for 5 min with rotation. Return the sample to the magnetic separation rack, wait 1-2 min for the solution to clear, and carefully remove the supernatant.
- 7.4.14. Add 1 mL of 1X TE Buffer, remove the sample tube from the magnetic separation rack. Without incubation, vortex briefly, return the sample to the magnetic separation rack, wait 1-2 min for the solution to clear, and carefully remove the supernatant.
- 7.4.15. Repeat step 7.4.14. Proceed immediately to step 7.5.3.

7.5. Elution of Chromatin from Antibody/Protein A/G Magnetic Beads and Reversal of Cross-links

➤ **Elution of Chromatin**

- 7.5.1. Remove and warm ChIP Elution Buffer in a 37°C water bath and ensure SDS is in solution. Set a water bath or thermomixer to 65°C.
- 7.5.2. Add 125 μ L of ChIP Elution Buffer to the 5% input sample tubes (from Step 7.4.3) and set aside at room temperature until Step 7.5.7.
- 7.5.3. Add 150 μ L ChIP Elution Buffer to each IP sample from Step 7.4.15.
- 7.5.4. Elute chromatin from the antibody/Protein A/G Magnetic Beads for 30 min at 65°C with gentle vortexing (1,200 rpm) every 5 min. A thermomixer works best for this step; however a 65°C water bath

with frequent mixing is also sufficient. Alternatively, elutions can be performed at room temperature with rotation, but may not be as complete.

7.5.5. Briefly centrifuge samples at 10,000 × g for 10 sec to remove evaporated sample from the microcentrifuge tube cap. Pellet Protein A/G Magnetic Beads by placing the tubes in a magnetic separation rack and wait 1 to 2 min for solution to clear.

7.5.6. Carefully transfer eluted chromatin supernatant (about 150 µL) to a new labeled tube.

➤ **Reversal of Cross-links**

7.5.7. To all tubes, including the 5% input samples from Step 7.5.2, reverse cross-links by adding 6 µL 5M NaCl and 2 µL RNase A (10 mg/mL), and incubate 30 min at 37°C. Then add 2 µL Proteinase K (20 mg/mL), mix thoroughly and incubate at 65°C for 4 h. This incubation can be extended overnight.

Immediately proceed to Step 7.6.1. Alternatively, samples can be stored at -20°C. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before DNA purification.

7.6. DNA Purification Using Spin Columns (Using ABclonal AFTSpin Multifunction DNA Purification Kit [RK30100] as an Example)

7.6.1. (Optional) Retrieve all sample tubes and equilibrate to room temperature. Add an equal volume of isopropanol and mix by vortexing.

7.6.2. Add 750 µL Buffer DB to each tube at a 1:5 ratio (sample volume : buffer volume). Mix thoroughly. If the total volume exceeds 550 µL, process in batches.

7.6.3. Transfer the solution to a Spin Column. Centrifuge at 12,000 × g for 1 min. Discard the flow-through and return the Spin Column to the empty collection tube.

7.6.4. Add 600 µL Buffer W1 to the Spin Column (ensure ethanol has been added as specified). Let stand for 2 min, then centrifuge at 12,000 × g for 1 min. Discard the flow-through.

7.6.5. Repeat Step 7.6.4 once.

7.6.6. Return the Spin Column to the empty collection tube. Centrifuge at 12,000 × g for 2 min to remove residual ethanol.

7.6.7. Transfer the Spin Column to a clean 1.5 mL tube. Open the lid and air-dry at 20–25°C for 2 min to remove residual ethanol.

7.6.8. Add 50 µL Nuclease-free Water (pre-warmed to 65–70°C for optimal elution) directly to the center of the spin membrane. Incubate at room temperature for 2 min, then centrifuge at 12,000 × g for 2 min. For higher DNA yield, reload the eluate onto the Spin Column and centrifuge for 1 min.

7.6.9. Discard the Spin Column. The eluate in the centrifuge tube is the purified DNA.

7.6.10. Proceed to DNA quantification using a Qubit fluorometer. Purified DNA can be stored at -20°C, used for library preparation, or analyzed by qPCR.

7.7. Quantification of DNA by qPCR

7.7.1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the positive control histone H3 sample, the negative control normal rabbit IgG sample, a tube with no DNA to control for contamination, and a serial dilution of the 5% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification.

7.7.2. Add 2 µL of the appropriate DNA sample and 6 µL of Nuclease-free Water to each tube.

7.7.3. Prepare a master reaction mix as described below. Setup 2-3 replicates for each PCR reaction. Add enough reagents to account for loss of volume. Add 12 µL of reaction mix to each PCR reaction tube or well.

Reagent	Volume
2X Universal SYBR Green Fast qPCR Mix	10 µL
RPL30 Primer(5 µM)	2 µL

7.7.4. Start the following PCR reaction program

Temperature	Time	Cycles
95°C	3 min	1
95°C	5 s	45
60°C	30 s	
Melt Curve	Default	

7.7.5. Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from each immunoprecipitation are expressed as a percent of the total input chromatin.

$$\text{Percentage of Input} = 5\% \times 2^{(C[T] \text{ Input Sample} - C[T] \text{ IP Sample})}$$

$$C[T] = CT = \text{Average threshold cycle of PCR reaction}$$

8. Appendix

8.1. Tissue processing:

8.1.1. Sample Collection

Grind tissues in liquid nitrogen or mince on ice, then transfer to 1.5 mL microcentrifuge tubes (100–150 mg per tube).

8.1.2. Freshly prepare Cell Fixation Solution (Final formaldehyde concentration is 1%)

Reagent	Volume
1X PBS Buffer (PBS)	1 mL
16% Formaldehyde Solution	62.5 μ L
Total Volume	~1 mL

Note: Prepare fresh and use immediately.

8.1.3. Add 1 mL of freshly prepared Cell Fixation Solution to the sample. Mix thoroughly by pipetting. Incubate on ice for 15 min, gently shaking periodically.

8.1.4. Stop cross-linking by adding 100 μ L of Glycine Solution (10X) to the sample. Mix and incubate on ice for 5 min,, gently shaking periodically.

Note: Pre-warm Glycine Solution (10X) to 37°C before use.

8.1.5. Centrifuge tissue at 4°C, 1,200 $\times$ g for 5 min. Discard the supernatant.

8.1.6. Wash cells two times with 1 ml ice-cold 1X PBS Buffer (PBS).

8.1.7. Centrifuge tissue at 1,200 $\times$ g for 5 min at 4°C, discard the supernatant.

Note: The experiment can be paused here. The tissue samples after removing the supernatant can be stored at -80°C. To proceed, continue to Step 7.2.

8.2. Troubleshooting Guide

Problem	Possible Causes	Recommendation
Concentration of the fragmented chromatin is too low.	Cell/nuclear lysis is incomplete.	If DNA concentration of the chromatin preparation is close to 200 μ g/mL, add additional chromatin to each IP to give at least 20 μ g/IP and continue with protocol.
	Not enough cells were used for the chromatin preparation.	Count a separate plate of cells before cross-linking to determine an accurate cell number.
Chromatin is under-fragmented and fragments are too large	Cells may have been over cross-linked. Prolonged crosslinking treatment	Shorten the crosslinking time to 10 minute.

(>900 bp).	(exceeding 10 minutes) may inhibit chromatin digestion efficiency.	
	Too many cells/tissues were processed.	Before crosslinking, count cells from a reserve plate to determine the exact cell number to be used. Refer to Appendix 1 for pre-experiment optimization of sonication conditions.
No product or very little product in the input PCR reactions.	Not enough DNA added to the PCR reaction or conditions are not optimal.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough chromatin added to the IP or chromatin is over-sonicated b	See recommendations for problem 1.
No product in the positive control Histone H3-IP RPL30 PCR reaction.	Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.	Be sure to add 20 µg of chromatin and 10 µg of antibody to each IP reaction and incubate with antibody for ≥3 hours. After adding Protein A/G beads, continue incubation for ≥1 hour.
	Incomplete elution of chromatin from Protein A/G beads.	Elution of chromatin from Protein A/G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
Quantity of product in the negative control Rabbit IgG-IP and positive control Histone H3-IP PCR reactions is equivalent.	Too much or not enough chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.	Add no more than 20 µg of chromatin and 2 µg of antibody to each IP reaction. Excessive chromatin or antibody amounts will increase nonspecific binding and elevate background signals.
	Too much DNA added to the	Add less DNA to the PCR reaction or

	PCR reaction or too many cycles of amplification.	decrease the number of PCR cycles. It is very important that the PCR products are analyzed within the linear amplification phase of PCR. Otherwise, the differences in quantities of starting DNA cannot be accurately measured.
No product in the Experimental Antibody-IP PCR reaction.	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough antibody added to the IP reaction.	Typically a range of 1 to 10 µg of antibody is added to the IP reaction; however, the exact amount depends greatly on the individual antibody.
	Antibody does not work for IP.	Increase the amount of antibody added to the IP. Find an alternate antibody source.

ABclonal Biotechnology Co. Ltd.

E-mail: info@abclonal.com

Web: www.abclonal.com

Headquarters: Building 5, Precision Medicine Industry Base Project I, Gaokeyuan 3rd
Road, Donghu New Technology Development Zone, Jiangxia District, Wuhan, Hubei, China