GENE CREATE

Plant Chromatin Immunoprecipitation (ChIP) Kit

Catalog#JKR23002P

Instruction Manual (For Two Groups)

Sufficient reagents for 6 ChIP assays per kit.
Store at -20 & 4°C

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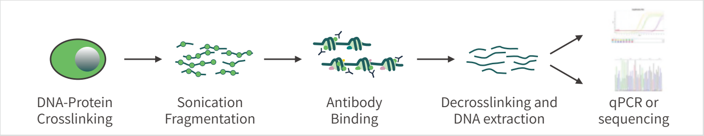
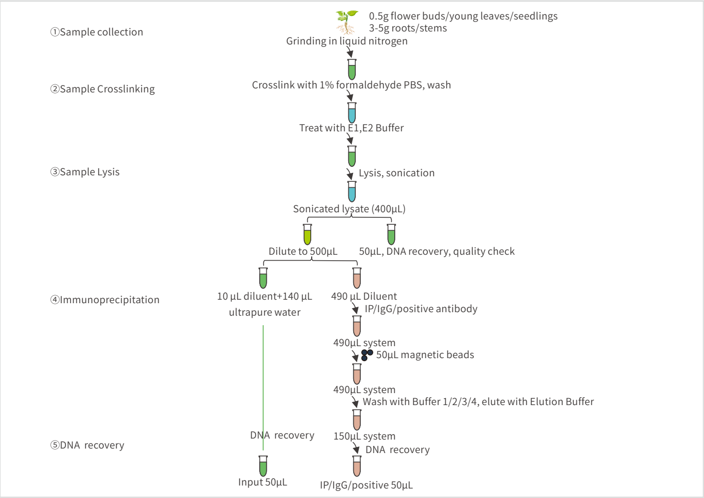
1. Experimental Principle

Chromatin Immunoprecipitation (ChIP) is used to study whether proteins and DNA interact in vivo. This technique helps researchers determine what histone modifications occur at specific locations in the genome within the nucleus. By leveraging the specific binding of antibodies to antigens, DNA fragments bound to the target protein are precipitated, accurately reflecting regulatory proteins bound to DNA sequences.

2. Experimental Procedure





3. Kit Components

Box A					
Component	**Volume (6T)**	**Storage Temperature**	**Component**	**Capacity (6T)**	**Storage temperature**
10×PBS	21mL	4°C	ChIP Buffer	7mL	4°C
Glycine Buffer	7mL	4°C	Protein A/G Magnetic Beads	350 μL	4°C
4× E1 Buffer	18mL	4°C	Elution Buffer	1mL	4°C
4×E2 Buffer	23mL	4°C	5M NaCl	150μL	4°C
Plant Lysis Buffer	4mL	4°C			

Box B					
Component	**Volume (6T)**	**Storage Temperature**	**Component**	**Capacity (6T)**	**Storage temperature**
Wash Buffer 1	7mL	-20°C	Normal Rabbit IgG	6μL	-20°C
Wash Buffer 2	7mL	-20°C	Normal Mouse IgG	6μL	-20°C
Wash Buffer 3	7mL	-20°C	Positive Antibody (Histone-H3)	6μL	-20°C
Wash Buffer 4	14mL	-20°C	Plant Protease Inhibitor (100×)	400μL	-20°C
Proteinase K	48μL	-20°C	RNase A	24μL	-20°C

Note: 6T refers to 6 individual immunoprecipitation experiments; the ChIP-qPCR procedure includes IgG, IP, and positive control (optional) groups, requiring 2-3T of reagents.

The kit does not include the following products:

- 37% formaldehyde or 16% formaldehyde
- DNA purification and recovery kit
- DNA Library Prep Kit
- Qubit fluorometer and quantification kit
- SYBR Green qPCR Mix
- Positive Primer

4. Operation Steps

4.1 Sample Crosslinking

a Buds/Tender Leaves/Seedlings

Preparation:

1×PBS: Take 500 μL of 10×PBS, add 4.5 mL of ultrapure water, mix well;

1% Formaldehyde PBS Solution: Take 27 μL of 37% formaldehyde solution, add 973 μL of 1× PBS, mix well;

E1 Buffer: Take 250 μL of 4×E1 Buffer, add 750 μL of ultrapure water, mix well;

E2 Buffer: Take 500 μL of 4×E2 Buffer, add 1.48 mL of ultrapure water and 20 μL of Plant Protease Inhibitor (100×), then mix well.

1) Place fresh or -80°C frozen samples (approximately 0.5 g) in a mortar, immerse in liquid nitrogen until brittle, then grind with liquid nitrogen, and transfer the powder to a 2 mL EP tube;
2) Add 1mL of 1% formaldehyde PBS solution, pipette to mix, and incubate with rotation at room temperature for 10-15 min;
3) Add 100 μL of Glycine Buffer, incubate with rotation at room temperature for 5 min, centrifuge at 3000g, 4°C for 5 min, and discard the supernatant;
4) Add 1 mL of pre-chilled PBS to resuspend the pellet by pipetting, centrifuge at 3000g, 4°C for 5 min, discard the supernatant, then add 1 mL of pre-chilled PBS again to wash once, for a total of two washes, and discard the supernatant;
5) Add 1 mL of E1 Buffer, vortex to mix, incubate on ice for 5 min, centrifuge at 3000g, 4°C for 5 min, and discard the supernatant;
6) Add 1 mL of E2 Buffer (containing protease inhibitors), vortex to mix, incubate on ice for 10 min, vortex every 3 min, after incubation let stand for 1-3 min, aspirate the upper suspension, discard the lower large particles, centrifuge the upper suspension at 3000g, 4°C for 5 min, and discard the supernatant;
7) Add 1 mL of E2 Buffer (containing protease inhibitors) again, vortex to mix, incubate on ice for 10 min, vortex every 3 min, centrifuge at 3000g, 4°C for 5 min, discard the supernatant (repeat this step if the supernatant remains unclear).

b Root/Stem

Preparation:

1×PBS: Take 3 mL of 10×PBS, add 27 mL of ultrapure water, mix well;

1% Formaldehyde PBS solution: Take 270 μL of 37% formaldehyde solution, add 9730 μL of 1× PBS, mix well;

E1 Buffer: Take 2.5 mL of 4×E1 Buffer, add 7.5 mL of ultrapure water, mix well;

E2 Buffer: Take 2.75 mL of 4×E2 Buffer, add 8.14 mL of ultrapure water and 55 μL of Plant

Protease Inhibitor(100×), mix well.

1) Place fresh or -80°C frozen samples (3-5g sample) in a mortar, immerse in liquid nitrogen until brittle, then grind with liquid nitrogen, and transfer the powder to a 50 mL centrifuge tube;
2) Add 10 mL of 1% formaldehyde PBS solution, pipette to mix, and incubate with rotation at room temperature for 10-15 min;
3) Add 1 mL of Glycine Buffer, incubate with rotation at room temperature for 5 min, centrifuge at 3000g, 4°C for 5min, and discard the supernatant;
4) Add 10 mL of pre-chilled PBS, vortex to mix, centrifuge at 3000g, 4°C for 5 min, discard the supernatant, then add 10 mL of pre- chilled PBS again to wash once, for a total of two washes, discard the supernatant;
5) Add 10 mL of E1 Buffer, vortex to mix, incubate on ice for 5 min, centrifuge at 3000g, 4°C for 5 min, discard the supernatant;
6) Add 10 mL of E2 Buffer (containing protease inhibitors), vortex to mix, incubate on ice for 10 min, vortex every 3 min, pass the suspension through a 200-mesh cell strainer or two layers of Miracloth filter cloth, centrifuge the filtrate at 3000g, 4°C for 5 min, discard the supernatant;
7) Add 1 mL of E2 Buffer (containing protease inhibitors), vortex to mix, transfer the liquid to a 2 mL EP tube, and incubate on ice for 10 min, vortexing every 3 min. Centrifuge at 3000g, 4°C for 5 min, and discard the supernatant. (If the supernatant remains unclear, repeat this step as needed.)

Note: The crosslinking temperature must always be kept below 25°C; formaldehyde concentration and crosslinking time vary for different sample types and can be adjusted according to established conditions.

4.2 Sample Lysis

Preparation:

Take 495 μL of Plant Lysis Buffer, add 5 μL of Protease Inhibitor (100X), and mix well. (If slight precipitation or turbidity is observed in the Lysis Buffer during use, this is normal; it can be used after warming to room temperature or heating at 37°C.)

1) Lysis: Add 400 μL of Plant Lysis Buffer to the cross-linked sample pellet from the previous step, and rotate-incubate at 4°C for 40– 60 min, or lyse by standing on ice for 40–60 min (depending on tissue type), vortexing to mix every 5 min;
2) Sonication: Perform low-temperature sonication for fragmentation (pre-experiments should be conducted to optimize conditions based on the sonicator type; reference conditions: 20% power, 5 s on, 2 s off, sonicate for 15–30 min, all in an ice bath). After sonication, centrifuge at 10,000 g, 4°C for 10 min, and collect the supernatant (this step can be paused, with samples stored at – 20°C);

   Note: Ultrasonic conditions need to be optimized for different tissue types. It is recommended to pre-optimize ultrasonic conditions for various samples before each chip experiment. A gradient quality control of ultrasonic times can be performed: after running at the shortest reference time, take 10μL samples every 5 min for 1% agarose gel electrophoresis to determine DNA fragment size.

3) Decrosslinking: Take 50μL supernatant, add 100μL ultrapure water and 1μL RNase A, mix well and incubate at 37°C for 5 min. Then add 6 μL 5M Nacl and 2 μL Proteinase K, and incubate at 65°C for 3 hours or overnight.
4) DNA Recovery: Use a DNA purification and recovery kit according to the instructions, and finally elute with 50μL ultrapure water (this sample can also serve as Input).
5) Quality Control: Determine DNA concentration using a Qubit fluorometer, and detect DNA fragment size by 1.5% agarose gel electrophoresis (optimal fragment range is 200-700 bp for ChIP-qPCR and around 200-500 bp for ChIP-seq).

4.3 Magnetic Bead Preparation (per IP reaction)

1) Remove Protein A/G Magnetic Beads from the 4°C refrigerator, invert and mix several times, and transfer 50 μL to a 1.5mL EP tube;
2) Add 200 μL of pre-chilled ChIP Buffer to resuspend the beads, place on a magnetic stand for 2 min, aspirate and discard the supernatant, and repeat this step once, for a total of two washes.

4.4 Immunoprecipitation (Single IP Reaction)

Preparation:

Based on experimental requirements, set up experimental groups. For ChIP-qPCR, groups are: Input, IgG, IP, and optional positive control, requiring 2-3 IP reactions. For ChIP-seq, groups are: Input and IP, requiring 1 IP reaction.

1) According to the DNA concentration measured by quality testing, dilute the sonicated lysate supernatant with ChIP Buffer to a final volume of 500 μL (dilute histone target DNA concentration to 10-20 μg/mL, transcription factor target DNA concentration to 20-40 μg/mL). Take 10 μL of the diluted sample add 140 μL of ultrapure water, and store as Input at -20°C for decrosslinking and recovery together with enriched samples.
2) Take 490 μL of the diluted sample, add 3-5 μg of the target antibody (IP group) or 1 μL of same-species IgG (IgG group) or 3 μL of positive antibody (positive group), and incubate with rotation at 4°C for 3 hours or overnight;
3) Retrieve the incubated sample, centrifuge briefly for 3 sec, and add it to the prepared magnetic bead tube, then incubate with rotation at 4°C for 2 hours;
4) Remove the sample from the silent mixer, place it on a magnetic stand for 2 min, and discard the supernatant;
5) Add 1 mL of Wash Buffer1 to resuspend the magnetic beads, place on a magnetic stand for 2 min, and discard the supernatant;
6) Add 1 mL of Wash Buffer2 to resuspend the magnetic beads, place on a magnetic stand for 2 min, and discard the supernatant;
7) Add 1 mL of Wash Buffer 3 to resuspend the magnetic beads, place on the magnetic stand for

2 min, and discard the supernatant;

8) Add 1 mL of Wash Buffer 4 to resuspend the magnetic beads, place on the magnetic stand for 2 min, discard the supernatant, repeat this step once, then briefly centrifuge for 3 sec, discard the supernatant to collect the pellet.

4.5 Elution and Recovery

Preparation:

Take the Elution Buffer from 4°C and allow it to reach room temperature until completely dissolved (may heat at 37°C to dissolve).

1) Add 150 µL of Elution Buffer to the magnetic bead pellet from the previous step, vortex to mix, incubate with rotation at room temperature for 15 min, briefly centrifuge for 3 sec, place on the magnetic stand for 2 min, and collect the supernatant;
2) Add 1 µL RNase A, mix well and incubate at 37°C for 5 min. Then add 6 µL 5M NaCl and 2 µL Proteinase K, incubate at 65°C for 3h (Input begins synchronous operation at this step);
3) Perform DNA purification and recovery using a kit according to the instructions, finally elute with 50 µL ultrapure water.

5. Experimental Analysis

5.1 ChIP-qPCR (Optional)

1) Reaction procedure: Add 2µL each of Input, IP, IgG, and positive sample into PCR reaction wells, with three replicate wells per sample. Add the remaining components according to the SYBR Green qPCR Mix instructions. After addition, label and centrifuge briefly for 10 sec, then place in a real-time PCR instrument for detection. Detailed spotting method is as follows ((the positive sample is only used with positive primers to confirm enrichment; positive primers need to be designed and synthesized independently: design primers based on housekeeping genes such as GAPDH/RPL30/Tubulin/Actin for the corresponding species):

		1	2	3	4	5	6	7	8	9	10	11	12
Positive primer	A	Input	Input	Input	IgG	IgG	IgG	IP	IP	IP	Positive	Positive	Positive
Primer1	B	Input	Input	Input	IgG	IgG	IgG	IP	IP	IP			
Primer2	C	Input	Input	Input	IgG	IgG	IgG	IP	IP	IP			
...	...	Input	Input	Input	IgG	IgG	IgG	IP	IP	IP			

2) Result Calculation
   $\Delta Ct\ [\text{normalized ChIP}] = (Ct\ [\text{ChIP}] - (Ct\ [\text{Input}] - \text{Log2}\ (\text{Input Dilution Factor}))$ $\text{Input Dilution Factor} = (\text{fraction of the input chromatin saved})^{-1}$
   Note: In our company's projects and kits, Input Dilution Factor = 50, $\log_2(50) = 5.644$
   $\Delta\Delta Ct\ [\text{ChIP/NIS}] = \Delta Ct\ [\text{ normalized ChIP }] - \Delta Ct\ [\text{IgG}]$
   $\text{Fold Enrichment} = 2^{(-\Delta\Delta Ct\ [\text{ ChIP/NIS }])}$
   $\%\text{Input} = 2\% \times 2^{(Ct[\text{Input}] - Ct[\text{ChIP}])}$

5.2 ChIP-seq (Optional)

1) Library construction: Take an appropriate amount of Input and IP samples, and proceed according to the DNA library preparation kit instructions.
2) Library quality control
3) Sequencing
4) Bioinformatics analysis

6. Frequently Asked Questions

Q1: How to explore ultrasonic conditions

1) Contact ultrasonic instrument: Take a 1.5 mL EP tube, add 0.4-0.6 mL of liquid, place the probe at the center of the EP tube, about two-thirds below the liquid surface, set the ultrasonic time to 5 sec, and gradually increase the power until bubbling begins—this power is the maximum ultrasonic power. Based on this, set three different power gradients and time gradients for exploration.
2) Non-contact type: Exploration can be conducted according to the manufacturer's recommendations.

Q2: Post-sonication fragments do not meet requirements

1) There are compliant fragments as well as large, concentrated fragments that cannot be sonicated, possibly due to excessive crosslinking temperature. Always maintain temperature below 25°C during crosslinking, especially in summer when temperatures are high; place reagents and samples at the air conditioning vent for a period before proceeding.
2) Fragments are highly concentrated and less than 200bp, indicating unsuitable sonication power and time; pre-experiments are needed to explore optimal sonication conditions.

Q3: No difference in Ct values between IP and IgG samples

1) Antibody failed to enrich DNA; try replacing the antibody.
2) IgG background is too high; increase wash cycles or reduce DNA input in the

immunoprecipitation step.

3) Binding site prediction error; primers need to be redesigned.

Q4: Abnormal melting curve

1) Melting curve shows non-single peak, possibly due to non-specific amplification or primer dimer formation, requiring primer redesign.

Q5: Sample DNA concentration is very low, below 10 ng/μL

1) Insufficient sample input: consider increasing initial sample input, especially for muscle, heart, etc.
2) Incomplete lysis: sample is over-crosslinked or insufficiently ground.
3) If encountering difficult-to-lyse samples, they can be rapidly frozen at -80°C after adding Lysis Buffer, thawed at 37°C, and subjected to three freeze-thaw cycles.

Simplified Operation Manual

1. Sample Cross-linking

1) Take an appropriate amount of sample, grind with liquid nitrogen, add 1 mL of 1% formaldehyde PBS solution, and incubate with rotation at room temperature for 10-15 min;
2) Add 100 μL of Glycine Buffer, incubate with rotation at room temperature for 5 min, centrifuge at 3000g, 4°C for 5 min, and discard the supernatant;
3) Wash twice with 1 mL of pre-chilled PBS;
4) Add 1 mL of E1Buffer, vortex to mix, incubate on ice for 5 min, centrifuge at 3000g, 4°C for 5 min, and discard the supernatant;
5) Add 1 mL of E2Buffer, vortex to mix, incubate on ice for 10 min, let stand for 1-3 min, then aspirate the upper suspension;
6) Add 1 mL of E2 Buffer again, incubate on ice for 10 min, centrifuge at 3000g, 4°C for 5 min, and discard the supernatant.

Note: For root/stem, increase the reaction system according to the instructions.

2. Sample Lysis

1) Add 400 μL of Plant Lysis Buffer (containing Protease Inhibitor), rotate and incubate at 4°C for 40-60 min, or lyse by standing on ice for 40-60 min, depending on the sample type;
2) Perform low-temperature sonication, then centrifuge at 10000g, 4°C for 10 min, and collect the supernatant (reference conditions: 20% power, 5 s on, 2 s off, sonicate for 15–30 min, all performed in an ice bath);
3) Take 50 μL of supernatant, add 100 μL of ultrapure water and 1 μL of RNase A, mix well and incubate at 37°C for 5 min, then add 6 μL of 5M NaCl and 2 μL of Proteinase K, and incubate at 65°C for 3 hours or overnight;
4) Perform DNA recovery, finally elute with 50 μL of ultrapure water;
5) Measure the concentration of the recovered DNA, and detect the DNA fragment size by 1.5% agarose gel electrophoresis.

3. Magnetic Bead Preparation

1) Take 50 μL of Protein A/G Magnetic Beads;
2) Wash the beads twice with 200 μL of pre-chilled ChIP Buffer.

4. Immunoprecipitation

1) Dilute the sample with ChIP Buffer, take 10 μL of the diluted sample, add 140 μL of ultrapure water, and store as Input at -20°C;
2) Take 490 μL of the diluted sample, add 3-5 μg of target antibody (IP group) or 1 μL of same-species IgG (IgG group) or 3 μL of positive antibody (positive group), and incubate with rotation at 4°C for 3 h or overnight;
3) After incubation, transfer to prepared magnetic bead tubes and incubate with rotation at 4°C for 2 h;
4) Wash the beads once each with Wash Buffer 1, Wash Buffer 2, and Wash Buffer 3, and wash twice with Wash Buffer 4.

5. Elution and Recovery

1) Vortex and mix 150μL of Elution Buffer, then rotate and incubate at room temperature for 15 min;
2) Add 1μL of Rnase A, mix well, and incubate at 37°C for 5 min. Then add 6μL of 5M NaCl and 2μL of Proteinase K, and incubate at 65°C for 3 hours (Input begins simultaneous operation at this step);
3) Recover DNA, eluting with 50μL of ultrapure water.

6. Experimental Analysis

1) ChIP-qPCR (Optional)
2) ChIP-seq (optional)

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