Product Information

G-Fast® Chromatin immunoprecipitation Kit

Catalog Number: f-CHIP-01 (15 Assays)

Description:
This kit is designed for rapid immunoprecipitation of chromatin from various cell types or tissues within 3-4 hours, to identify the special proteins associated with the DNA regions (chromatin).
The kit provides an innovative technology and a fast, simple procedure for nuclei isolation, immunoprecipitation, DNA isolation and purification, able to selectively isolate the interested protein-DNA complexes including the specific modified histones, transcription factors or co-factors, suitable for working on a small amount of cells (10e5)/tissues (10mg) to the large amount of samples (up to 500mg)

Kit contains:

Component	Quantity (15 assays)	Storage
Zmtech Cytoplasmic Lysis Buffer (C207020, clear cap)	1.5 mL	2-8°C
Zmtech Nuclei Isolation Buffer (D207040, yellow cap)	150 uL	2-8°C
Zmtech IP Washing Buffer (P209020, purple sticker)	8.0 mL	2-8°C
Zmtech IP Buffer (P209010, green sticker)	25.0 mL	2-8°C
Zmtech Protein A/G (P209030, red cap)	500 uL	2-8°C
DNA Isolation Reagent-A (D21010, blue cap)	1.0 mL	-20°C
DNA Isolation Reagent-B (D21020, clear cap)	50.0 uL	-20°C
DNA Isolation Reagent-C (D21030, pink cap)	1.0 mL	-20°C
DNA Precipitation Buffer (PS-01D, orange cap)	1.5 mL	-20°C
DTT, 1M (Dissolved in 0.1 ml ddH_2O)	1 vial	–20°C
Protease/Phosphatase Inhibitors (I208052)	600 uL	–20°C

supplied in DMSO, contains optimized AEBSF, Aprotinin, E64, Leupeptin, Pepstatin A, Sodium fluoride, Sodium orthovanadate and Sodium pyrophosphate.

Protocol: (Keep all buffers and cell/tissue samples on ice)

Prepare working reagents and other required solutions prior to proceeding

For 5 CHIP assays: (50-100mg tissues/ 10e7 cells per assay)	
Zmtech Cytoplasmic Lysis Buffer (500 ul)	add 0.5ul (1M DTT) and 10ul Protease/phosphatase Inhibitors (I208052)
Zmtech IP Washing Buffer (2.5ml)	add 2.0ul (1M DTT) and 30ul Protease/phosphatase Inhibitors (I208052)
Zmtech IP Buffer (8.0ml)	add 4.5ul (1M DTT) and 150ul Protease/phosphatase Inhibitors (I208052)
37% Formaldehyde solution (150 ul)	
1M Glycine solurtion (625 uL)	
12ug Antibodies (Interested antibodies, anti-RNA Polymerase II or anti-acetyl histone H3 antibody, normal rabbit IgG)	

1. **Cross-link protein-DNA and Quenching:**(performing this step under ventilation hoods)

- Add 150ul of 37% Formaldehyde directly into the cell culture wells/dishes/flasks containing 5ml media (approximately 50mg-100mg tissues or 1-10x 10e6 cells), and incubate for 10 minutes at room temperature with gentle shaking.
- Add 625ul of ice cold 1M glycine solution and incubate for 5 minutes at room temperature with gentle shaking .

2. **Isolate Nuclei:**

- Wash cells/tissues once with 5ml ice cold 1xPBS and aspirate liquids.
- Add 2ml ice cold 1xPBS into the wells/dishes/flasks and scrape cells/tissues into a clean 2.0 ml eppendorf tube. Centrifuge at 1,600 rpm for 5 minutes, aspirate liquids.
- Resuspend pellet with 500ul cytoplasmic lysis buffer by pipette up and down several times and incubate on ice for 10 minutes.

- Add 30ul Nuclei isolation buffer into the suspension, vortex at highest speed for 10 seconds. Immediately centrifuge at 5,000 xg for 1 minute at 4°C. Aspirate liquids.
- Resuspend pellet with 400ul IP buffer by pipette up and down several times and incubate on ice for 5 minutes.

3. **Sonication homogenization:**
   Using a pre-washed, clean sonicator, at 20% power speed for 15-20 seconds, repeated 10 times. Keep tubes on ice and wait 30-40 seconds between each pulse to avoid over-heat the samples during sonication. (*Optimize the shearing conditions to obtain 200-1000bp DNA fragments and avoid foaming formation or over-sheared DNA fragments.)

4. **Immunoprecipitation (IP):**

- Centrifuge the sonicated samples at 10,000 xg for 10 minutes at 4°C and transfer all the supernatant into a new tube. Save 25ul for DNA input control and perform the DNA isolation and purification with **step 11.** Store the supernatant at -80°C or setup the IP assays as following table:

IP Assays	Tube 1.	Tube 2.	Tube 3.	Tube 4.	Tube 5.	Tube 6.	Tube 7.
Supernatant	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul	50 ul
IP Buffer	200 ul	200 ul	200 ul	200 ul	200 ul	200 ul	200 ul
Antibodies	2 ug	10 ug	2 ug	10 ug	2 ug	2 ug	No
	Interested antibody-1		Interested antibody-2		Anti-RNA Polymerase II (Positive Control)	Normal rabbit IgG (Negative Control)	No antibody (Negative Control)

- Add 200ul IP buffer into each IP tube containing 50-100ul supernatant.
  Positive control IP:**(Optional)** add 2ug of anti-RNA Polymerase II antibody or anti-acetyl histone H3 antibody. Note:the PCR reaction can be carried out to detect GAPDH promoter using the control primers.
  Negative control IP: add 2ul of normal rabbit IgG (1mg/ml) or not add antibody.
  IP assays (2-4 antibodies): add 2-10ug of interested antibody. Do duplication of IPs with two different concentrations of antibody for maximizing the antibody-Protein binding. **(recommend)**

5. Incubate in an ultrasonic water bath for 15 minutes at 4°C or incubate on a rocking platform for 1.5 hours at room temperature (or overnight at 4°C) with gentle shaking.
6. Centrifuged at 10,000xg for 10 minutes at 4°C, and transfer the supernatant into the new clean tubes containing 30ul of protein A/G beads.
7. Incubate on a rocking platform for 45 minutes at 4°C with gentle shaking, and then centrifuge at 2500 xg for 10 minutes at 4°C. Aspirate liquids.
8. Add 500ul IP buffer to resuspend the pellet by gentle pipette up and down with 1.0ml pipette tip (Don't vortex) and centrifuge at 5,000 xg for 5 minutes at 4°C. Aspirate liquids.
9. Add 500ul IP washing buffer to resuspend the pellet by gentle pipette up and down with 1.0ml pipette tip (Don't vortex) and centrifuge at 5,000 xg for 5 minutes at 4°C. Aspirate liquids.
10. Add 500ul IP buffer to resuspend the pellet by gentle pipette up and down with 1.0ml pipette tip (Don't vortex) and centrifuge at 5,000 xg for 5 minutes at 4°C. Aspirate liquids.
    (The remained unbound proteins and non-specific DNA fractions were washed out).

11. DNA isolation:

- Resuspend the IP pellet with 50ul DNA isolation reagent-A and 2.5ul DNA isolation reagent-B. Incubate at 60°C for 10 minutes.
- Place the tube in a PCR machine (or water bath or block) and incubate at 95°C for 10 minutes.
- Centrifuge at 12,000 xg for 2 minutes at 4°C and transfer the supernatant into a clean tube.
- Add 50ul DNA isolation reagent-C into the supernatant and mix thoroughly by vortexing.
- Centrifuge at 5,000 xg for 5 minutes at 4°C and transfer the supernatant into a clean tube.

- Add 100ul DNA precipitation solution (Cat.#: PS-01D) into the supernatant. Mix well and centrifuge at 12,000 xg for 10 minutes at 4°C. Aspirate liquid.
- Simply wash DNA pellet with 80% ethanol for 2 times (don't resuspend the DNA pellets). Air-dry pellet for 5-10 minutes and dissolve DNA in 50ul TE buffer or distilled water. Measure the DNA Concentration with 260/280nm and store DNA at -20°C.
- Pipette 1-5ul DNA solution into a 25ul PCR Master Mixture and run PCR/Real-Time PCR at thermal cyclers.

Flow Chart of CHIP assay: (an innovative nuclei isolation/DNA purification technology)

1. **Cross-link and quenching** (15mins)
2. **Nuclei preparation and Sonication** (30mins)
3. **Antibodies Immunoprecipitation (IP)** (2.5 hrs)
4. **Cross-link reversal and DNA purification** (30mins)
5. **qPCR quantitation** (1.5 hrs)

Cells (10e7)/ Tissues (50mg) in 5ml media

Cross-link Protein-DNA Quenching
Add 1% Formaldehyde, RT, 10 minutes
Add 125mM Glycine, RT, 5 minutes

Cell/tissue collection

Nuclei isolation
Add Cytoplasmic lysis buffer (500ul)
Add Nuclei isolation buffer (30ul)
Spin 10 minutes

Pellet -------- Supernatant (discard)
(Nuclei) (Cytoplasmic proteins/DNA)

Add PI buffer (400ul)
Sonication homogenization
Spin 10minutes

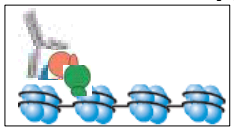
Supernatant
(Nuclear protein-DNA complex)

Setup the IP assays

Input control	**Positive control**	**Negative control**	**IPs**
(25ul)	(50ul)	(50ul)	(4x 50ul)
	anti-RNA Poly. II	normal Rabbit IgG	Interested antibodies

Add IP buffer (200ul)
Add antibodies (2ug-10ug)
Spin 10 minutes
Add Protein A/G beads, 45 minutes
Spin 10 minutes

Pellets (protein A/G-antibody-protein-DNA complex)



Wash-1,-2,-3 with IP buffer (500ul)

DNA isolation and purification

Cross-link reversal
Add DNA Isolation reagent A (50ul) and B (2.5ul)
Incubate 60°C for 10 minutes, 95°C for 10 minutes
Add DNA isolation reagent C (50ul)
Add DNA precipitation solution (100ul), spin 10 minutes
Wash DNA pellet with 80% ethanol twice, Air-dry
Dissolve DNA with ddH_2O or TE buffer

Quantitative PCR or Southern Blots

Technical highlights:

- Nuclei isolation is an important step for removing the cytoplasmic proteins, cytosolic DNA and other organelles, able to reduce IP backgrounds and increase the antibody-protein binding.
- Sonication Optimizations: avoid too much foaming formation and overheat the samples by changing either the power settings or/and the number of pulses. Keep samples on ice and wait for 30-60 seconds between sonication pulses. Run input DNA control on 2% agarose gel to visualize the shearing efficiency. The ideal sheared DNA fragments should be around 500-700bp.
- Antibodies-protein binding and qPCR Optimizations: do duplicated IP assays using 2ug and 10ug of antibody to optimize the binding conditions and maximize the yields of specific immuno-complexes. Do duplicated qPCR reactions using 1ul and 5ul of DNA to quantify DNA signals.
- An innovative, fast DNA purification procedure (30 minutes): No toxic chemical involved (e.g. phone-chloroform) and no spin column purification steps are able to avoid the loss of targeted DNA during binding and washing proceeding.

Additional information:

- Test 1: using input DNA control to run on 2% agarose gel for optimizing the sonication conditions.
- Test 2: add protein loading buffer to immune complex, boil for 10 minutes and run on acrylamide gel for evaluating IP results.
- Design targeted primers specific for the interested genes and control primers specific for the GAPDH promoter.
- Preparation of PCR Master Mix for a single reaction (total volume: 25uL) in a PCR microtube or plate.

Components	Volume (µL)	Final Concentration
All-IN-One qPCR Mixture(2x)	12.5	1x
$MgCl_2$	X	(3mM-7mM)
DNA Template	2	(100pg-500pg/µL)
Forward primer (5µM)	1	200nM
Reverse primer (5µM)	1	200nM
Probes or SybrGreen	1	70nM
PCR grade water	up to 25 µL	

Setup typical thermal cycling parameters

Enzyme activation Step:	95°C	5 minutes
25-40 Cycles:		
Denaturation	95°C	15 seconds
Annealing	X°C	30 seconds dependent on Tm of primers
Extension	60°C	30 seconds (acquire data at the end of this step)

Precautions and Disclaimer:

This product and procedure described are intended for R&D use only. Purchase of this product does not convey a license to perform any patented process.

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