Part One:

After stimulation, the cells are washed, and collected for CHIP experiment.

Preparation: Thaw 200× Protease Inhibitor Cocktail (PIC) and 10× Glycine Solution; prepare 2mL PBS + 10 μl 200× PIC for each cell sample (about $1×10^7$ grade, 15cm petri dish) on ice; prepare 40 mL PBS for each cell sample (about $1×10^7$ grade) on ice for later use; prepare 540 μl 37% formaldehyde for each cell sample (about $1×10^7$ grade) and store it at room temperature for later use;

1) Cross-link the protein of STAT3 to the DNA, and add 540 μl 37% formaldehyde to each 15cm petri dish containing 20mL medium. For suspended cells, add 540 μl 37% formaldehyde to the cells suspended in 20mL of medium, vortex briefly, and incubate for 10 minutes at room temperature.

2) Add 2mL 10× glycine to each 15cm petri dish containing 20mL medium, vortex briefly to mix, and incubate at room temperature for 5 minutes.

3) For suspended cells, transfer the cells to a 50mL conical tube, centrifuge 500×g at 4°C for 5 minutes, and wash the pellet twice with 20mL ice-cold PBS. Remove the supernatant and proceed to the next step.

4) For adherent cells, remove the culture medium and wash the cells twice with 20mL of ice-cold 1× PBS, thoroughly removing the washing solution from the culture dish each time.

5) Add 2 mL ice-cold PBS + PIC to each 15cm petri dish; scrape off the cells, centrifuge at 2,000×g at 4°C for 5 minutes, remove the supernatant, and proceed to the next step.

Part Two: Nuclear preparation and chromatin digestion

Preparation: Thaw 200× Protease Inhibitor Cocktail (PIC); prepare 1M DTT (192.8 mg DTT + 1.12 mL dH_2O); prepare 1 mL 1× Buffer A (250 μl 4× Buffer A + 750 μl water) + 0.5 μl for each IP reaction 1M DTT + 5 μl 200× PIC stored on ice for later use; prepare 1.1 mL 1× Buffer B (275 μl 4× Buffer B + 825 μl water) + 0.55 μl 1M DTT for each IP reaction; prepare 100 μl 1× ChIP Buffer (10 μl 10× ChIP Buffer + 90 μl water) + 0.5 μl 200× PIC;

1) Resuspend the cells and added 1 mL ice-cold 1× Buffer A + DTT + PIC per IP,

incubate on ice for 10 minutes, invert the tube every 3 minutes to mix.

2) Centrifuge 2000×g at 4°C for 5 minutes, remove the supernatant, and resuspend the pellet. Add 1 mL ice-cold 1× Buffer B + DTT for each IP, and centrifuge again. Then, each IP was resuspended the pellet with 100 μl ice-cold 1× Buffer B + DTT, and transferred to a 1.5 mL centrifuge tube.

3) Add 0.5μl Micrococcal Nuclease per IP, incubate at 37°C for 20 minutes, mix frequently to digest DNA with a length of 150-900bp, and mix every 3 to 5 minutes.

4) Add 10 μl 0.5M EDTA on ice for 1-2 minutes to stop digestion.

5) Centrifuge 16000×g at 4°C for 1 minute and remove the supernatant.

6) Resuspend with 100 μl of 1× ChIP Buffer + PIC and incubate on ice for 10 minutes.

7) Concentrate each IP resuspension of the same sample in one EP tube and ultrasonically break last 15 seconds for 20 times.

8) Centrifuge 9400×g at 4°C for 10 minutes.

Transfer the supernatant to a new EP tube and store in a refrigerator at -80°C.

Part Three: Chromatin immunoprecipitation

Preparation: Thaw 200× Protease Inhibitor Cocktail (PIC) and 10× ChIP Buffer; Thaw chromatin preparations and store on ice; prepare low salt wash and store at room temperature; prepare high salt wash and store at room temperature.

1) Prepare enough 1× ChIP Buffer according to the number of reaction IPs on one tube. Prepare 400 μl 1× ChIP Buffer (40 μl of 10× ChIP Buffer + 360 μl water) + 2 μl 200× PIC for each IP, and store on ice.

2) Add 100 μl digested cross-linked chromatin preparation to the prepared 1× ChIP Buffer.

3) Transfer 10μl of the diluted chromatin sample into a microcentrifuge tube as a 2% input group and store at -20°C until further use.

4) For each IP reaction, transfer 500 μl of diluted chromatin to a 1.5 mL microcentrifuge tube and add 5μg of immunoprecipitation antibody, including the negative control antibody and the target antibodies of STAT3, and incubate overnight at 4°C on a shaker.

5) Resuspend ChIP-grade protein G magnetic beads, immediately add 30μl protein G

magnetic beads to IP reaction, and incubate for 2 hours on a shaker at 4°C.

6) Place IP reaction tube on the magnetic separation rack and remove the supernatant.

7) Add 1 mL low salt wash to the protein G magnetic beads, incubate for 5 minutes on a shaker at 4°C, repeat steps 6 and 7 twice, with three washes.

8) Add 1 mL high salt wash and incubate for 5 minutes on a shaker at 4°C.

9) remove the supernatant by magnetic separation rack and proceed to the next step.

Part four: Elution of chromatin and crosslinking inversion from antibody/protein G magnetic beads

Preparation: 37°C water bath 2× ChIP Elution Buffer to ensure that SDS is dissolved; set the water bath to 65°C; prepare 150 μl 1× ChIP Elution Buffer (75 μl 2× ChIP Elution Buffer + 75 μl water).

1) Add 150 μl 1× ChIP Elution Buffer to the 2% input group and store at room temperature.

2) Add 150 μl 1× ChIP Elution Buffer to each IP.

3) The chromatin was eluted with antibody/protein G magnetic beads at 65°C for 30 minutes.

4) Place the magnetic beads on the magnetic separation rack and wait for 1 to 2 minutes for the solution to clear.

5) Carefully transfer the eluted chromatin supernatant to a new tube.

6) Add 6μl 5M NaCl and 2 μl proteinase K to all test tubes for reverse crosslink, and incubate at 65°C for 2 hours.

Part Five: Purify DNA using spin columns

1) Add 750 μl DNA Binding Buffer to each DNA sample and vortex briefly.

2) Transfer 450 μl of each sample to the DNA spin column in the collection tube.

3) Centrifuge at 18,500×g for 30 seconds.

4) Take out the spin column from the collection tube, discard the liquid, and put the collection tube back into the spin column.

5) Transfer the remaining 450 μl of each sample to the spin column in the collection tube. Repeat steps 3 and 4.

6) Add 750 μl of DNA Wash Buffer to the spin column in the collection tube.

7) Centrifuge at 18,500×g for 30 seconds.

8) Take out the spin column from the collection tube, discard the liquid, and put the collection tube back into the spin column.

9) Centrifuge at 18,500×g for 30 seconds.

10) Discard the collection tube and liquid, and save the spin column.

11) Place the spin column in a new 1.5 mL centrifuge tube and add 50 μl DNA Elution Buffer.

12) Centrifuge at 18,500×g for 30 seconds to elute DNA.

13) Take out and discard the spin column, the eluate is now purified DNA, and the sample can be stored in a refrigerator at -20°C.

Part Six: QPCR

The reaction system for Real time PCR amplification is 20μl (DBI Bestar® SybrGreen qPCR master Mix), and the reaction system is prepared according to the following table:

Bestar® SybrGreen qPCR master Mix	10
Forward Primer（10μM）	0.5
Reverse Primer（10μM）	0.5
DNA template	2
ddH_2O	7
Total	20 μl

PCR reaction conditions: 95°C for 2min, 94°C for 20s, 58°C for 20s, 72°C for 20s, 40 cycles.

Melting curve analysis: 94°C 30s, 65°C 30s, 94°C 30s

Repeat each sample 3 times.