Chromatin Immunoprecipitation 实验报告

一、实验器材

名称	厂家	型号
酶标检测仪	Rayto	RT-6100
台式高速冷冻离心机	DRAGONLAB	D3024R
掌上离心机	Wanwu	D1008E
涡旋混合器	Wanwu	MX-F
磁力搅拌器	Wanwu	MS-PB
脱色摇床	Wanwu	TSY-B
垂直电泳仪	Wanwu	BV-2
转印电泳仪	姜堰市天力医疗器械厂有限公司	BT-2
水浴锅	Wanwu	TL-420D
暗匣	Wanwu	WGA0017
暗室专用红灯	Wanwu	WGA0016
柯达胶片	ABI	WGJP0001
荧光定量 PCR 仪	苏净安泰	Stepone plus
超净工作台	上海天能科技有限公司	SW-CJ-1FD
凝胶成像系统	sigma	Tanon-1600R
制冰机	EPSON	SPR 80
扫描仪	Alpha Innotech	V370
灰度分析软件	Adobe	alphaEaseFC
图像分析软件	宁波新艺超声设备有限公司	Adobe PhotoShop
细胞超声波破碎仪		JY 92-IIN

二、实验试剂

试剂	厂家	货号
IP 裂解液	Wanwu	G2038-100ML
蛋白 A/G-beads	Millipore	IP05
50*cocktail	Wanwu	G2006
PMSF（100mM）	Wanwu	G2008
磷酸化蛋白酶抑制剂	Wanwu	G2007
BCA 蛋白定量检测试剂盒	Wanwu	G2026

5*/2*蛋白上样缓冲液	Wanwu	G2013/G2031
SDS-PAGE 凝胶制备试剂盒	Wanwu	G2003
蛋白 Marker	Wanwu	26617
TRIS	Wanwu	G5009-50
甘氨酸	Wanwu	G5010-500G
SDS	Wanwu	G5037-100G
PVDF 膜 0.45um	Wanwu	G6015-0.45
脱脂奶粉	Wanwu	G5002-100G
TWEEN 20	Wanwu	WGT8220
ECL	Wanwu	G2014
显影定影试剂	Wanwu	G2019
HRP 标记山羊抗兔	Wanwu	GB23303
HRP 标记驴抗山羊	Wanwu	GB23404
HRP 标记山羊抗小鼠	Wanwu	GB23301
HRP 标记山羊抗大鼠	Wanwu	GB23302
转移缓冲液	Wanwu	G2017
电泳缓冲液	Wanwu	G2018
TBS 缓冲液	Wanwu	G0001-2L
琼脂糖 Agarose	Wanwu	G5056
无水乙醇	国药集团化学试剂有限公司	10009218
2×SYBR Green qPCR Master Mix (High ROX)	Wanwu	G3322

三、CHIP 实验步骤

第一天：

（一）细胞的甲醛交联与超声破碎

1、取出 1 平皿细胞，测量培养基体积，按照终浓度为 1%的比例加入 37%甲醛；

2、37℃孵育 10min；(恒温培养箱)

3、终止交联：按照终浓度为 125mM 的比例加 2.5M 甘氨酸于平皿中。混匀后，在室温下放置 5min 即可；

4、吸尽培养基，用冰冷的 PBS 清洗细胞 3 次；

5、用 PBS 将细胞刮下来，2000g 离心 5min，去上清；

6、加入含蛋白酶抑制剂的 IP 裂解液裂解细胞（裂解液的量视细胞沉淀量而加）；

7、冰上充分裂解 30min，过程中用枪反复吹打细胞（或者放在漩涡混匀器上震荡），使其充分裂解；

8、超声破碎：宁波新芝 JY 92-IIN，2#探头，30%功率，4.5S 超声，10.5S 间隙；5min，总

计超声次数 20 次。超声过程中请一定注意要保持样品处于冰浴中，避免超声出现泡沫，并且处于较低温度；

（二）除杂及抗体哺育

1、超声破碎结束后，12000rpm， 4℃，离心 10min。去除不溶物质，取上清；**留取 90μl 做 input**，其余保存于－80 度。(取样①input 实验)

2、取 40μl 留作 Input 的超声破碎产物，加入 10μl5*还原型蛋白上样缓冲液，加热变性后进行 WB 检测，确认样品中含有目标蛋白。剩下 50μl 产物加入 5μl 的蛋白酶 K 和 2μl 5M NaCl（NaCl 终浓度为 0.2M），55℃过夜解交联；

3、解交联结束后，测核酸浓度，取部分样品进行 PCR 扩增，然后跑琼脂糖电泳，检测超声破碎的效果和确认样品中含有目标 DNA；

4、Input 结果确认后，取冻存在-80℃冰箱中的超声破碎产物 100μl，加入 900μl 含 1mM-PMSF 的 ChIP Dilution Buffer 和 20μl 的 50×PIC （cooktail）。再各加入 60μl Protein A +G Agarose/Salmon Sperm DNA。4℃颠转混匀 1h；

5、1h 后，在 4℃静置 10min 沉淀，4000rpm 离心 5min；

6、将样品分装至两支 1.5mL EP 管，一管中加入目的蛋白 IP 抗体 1μg，另一管中加入对应种属的 IgG 1μg。4℃颠转过夜；

第二天：

（一）免疫复合物的沉淀及清洗

1、孵育过夜后，每管中加入 200μl Protein A+G Agarose/Salmon Sperm DNA，4℃颠转 2h；

2、4℃静置 10min 后，4000rpm 离心 1min。除去上清；

3、依次用下列溶液清洗沉淀复合物。清洗的步骤：加入溶液，轻轻颠倒， 4000rpm 离心 3min，去除上清；

洗涤溶液：a. low salt wash buffer----one wash

b. high salt wash buffer-----one wash

c. LiCl wash buffer------one wash

d. TE buffer------two wash

注：完成上述所有洗涤步骤后所获得的沉淀即可用于 Western 检测，在完成所有的洗涤步骤后，取 30μl 样品加入 30μl SDS-PAGE 蛋白上样缓冲液(1X)，沸水浴煮沸 10 分钟后上样进行 WB；**（取样②IB 实验）**

4、现配 elution buffer（0.5gSDS+0.42gNaHCO3+50ml 水），加 200μl 至 EP 管；

5、加 5M NaCl 8μL，Protease K 20μL，55℃过夜解交联；

第三天：

（一）DNA 样品的回收（离心柱法）

1、取解交联后样本（体积为 200μl），加入 200 μl 缓冲液 GB，充分颠倒混匀，70℃放置 10min，简短离心以去除管盖内壁的水珠；

2、加入 200 μl 无水乙醇，充分振荡混匀 15s，简短离心以去除管盖内壁的水珠；

3、将上一步所得溶液和絮状沉淀都加入一个吸附柱 CB3 中（吸附柱放入收集管中），12000

g 离心 30s，倒掉废液，将吸附柱 CB3 放回收集管中；

4、向吸附柱 CB3 中加入 500 μl 缓冲液 GD（已加入无水乙醇），12000 g 离心 30s，倒掉废液，将吸附柱 CB3 放回收集管中；

5、向吸附柱 CB3 中加入 700 μl 漂洗液 PW（已加入无水乙醇），12000 g 离心 30s，倒掉废液，将吸附柱 CB3 放回收集管中；

6、向吸附柱 CB3 中加入 500 μl 漂洗液 PW，12000 g 离心 30s，倒掉废液；

7、将吸附柱 CB3 放回收集管中，12000 g 离心 2min，倒掉废液，室温放置 10min，以彻底晾干吸附材料中残余的漂洗液；

8、将吸附柱 CB3 转入一个干净的离心管中，向吸附膜的中间部位悬空滴加 50 μl TE 洗脱缓冲液，室温放置 5min，12000 g 离心 2min，将溶液收集到离心管中；

9、将离心得到的溶液再加入吸附柱 CB3 中，室温放置 2min，12000 g 离心 2min，将溶液收集到离心管中；

（二）分析沉淀所得 DNA（选用 RT-PCR 法或测序法）

1、RT-PCR 法

1.1、利用 JASPAR 等数据库预测转录因子结合位点，根据该结合位点设计引物并合成；

1.2、取 0.2ml PCR 管，配制如下反应体系：

2× qPCR Mix	10μl
2.5μM 基因引物	2μl
模板	2μl
ddH2O	6μl1

1.3、PCR 扩增

预变性	95℃，10min
循环（40 次）	95℃，15s→60℃，60s
熔解曲线	60℃→95℃，每 15s 升温 0.3℃。

1.4、扩增结束后，取产物跑胶，检测片段大小是否正确。

2、测序法

收集所得 DNA，测浓度后送往测序公司。

Chromatin Immunoprecipitation protocol

1、Experiment instruments

Name	Company	Model number
Microplate Reader	Rayto	RT-6100
Homogenizer	Wanwu	KZ-II
Centrifuge	DRAGONLAB	D3024R
Mini Centrifuge	Wanwu	D1008E
Vortex mixer	Wanwu	MX-F
Magnetic stirrers	Wanwu	MS-PB
Decolorizing shaker	Wanwu	TSY-B
Thermostatic Water Bath	Jiangyan Tianli Medical Devices Co., Ltd.	TL-420D
Camera obscura	Wanwu	WGA0017
Darkroom lamp	Wanwu	WGA0016
Photographic film	Wanwu	WGJP0001
Real-time qPCR	ABI	Stepone plus
Bechtop	AIRTECH	SW-CJ-1FD
Gel imaging system	Shanghai Tianneng Technology Co., LTD	Tanon-1600R
Ice Maker	sigma	SPR 80
Scanister	EPSON	V370
Gray analysis software	Alpha Innotech	alphaEaseFC
Image-analysis software	Adobe	Adobe PhotoShop
Ultrasonic Cell Disruptor	Ningbo Xinyi ultrasonic Equipment Co. LTD	JY 92-IIN

2、Reagents

Reagents	Company	Catlog
IP Lysis Buffer	Wanwu	G2038-100ML
Protein A/G-beads	Millipore	IP05
50*cocktail	Wanwu	G2006
PMSF（100mM）	Wanwu	G2008
Phosphoproteinase inhibitor	Wanwu	G2007
BCA protein quantitative detection kit	Wanwu	G2026
5*/2*loading buffer	Wanwu	G2013/G2031

SDS-PAGE kits	Wanwu	G2003
Protein Marker	Wanwu	26617
TRIS	Wanwu	G5009-50
Glycine	Wanwu	G5010-500G
SDS	Wanwu	G5037-100G
Milk	Wanwu	G5002-100G
TWEEN 20	Wanwu	WGT8220
ECL	Wanwu	G2014
Developer and fixer	Wanwu	G2019
PeroXidase-conjugated Goat Anti- Rabbit IgG(H+L)	Wanwu	GB23303
PeroXidase-conjugated Donkey Anti-Goat IgG(H+L)	Wanwu	GB23404
PeroXidase-conjugated Goat Anti-Mouse IgG(H+L)	Wanwu	GB23301
PeroXidase-conjugated Goat Anti-Rat IgG(H+L)	Wanwu	GB23302
Transfer buffer	Wanwu	G2017
Electrophoresis buffer	Wanwu	G2018
TBS buffer	Wanwu	G0001
Agarose	Wanwu	G5056
Anhydrous ethanol	Sinopharm Group Chemical Reagent Co. LTD	10009218
2×SYBR Green qPCR Master Mix (High ROX)	Wanwu	G3322

3、Protocol For ChIP

Day 1:

3.1 Formaldehyde cross-linking and ultrasonic disruption of cells

3.1.1. For cells: measure the volume of the medium, and add 37% formaldehyde until a final concentration of 1% is reached.

3.1.2. Incubate for 10 minutes at 37 ℃

3.1.3. Terminate the cross-linking: Block the reaction with 500 µl Glycine 2.5 M (final concentration 125 mM). Incubate for 5 minutes at room temperature.

3.1.4. Discard the medium and wash the cells 3 times with ice cold PBS;

3.1.5. Using a cell scraper scrape off the cells in PBS, centrifuge at 2000g/min for 5min, and then

discard the supernatant;

3.1.6. Breaking the cells in IP Lysis Buffer supplemented with protease inhibitor(the volume of IP Lysate Buffer depends on the amount of cells).

3.1.7. To facilitate the cell membrane breaking，the cells were repeatedly blown with a pipette (or shaken on a vortex mixer);

3.1.8. Ultrasonic Crushing: Ningbo Xinzhi JY 92-IIN, 2# probe, 30% power, 4.5s ultrasound, 10.5s interval， 5min, and 20 times. During the ultrasonic process, please be careful to keep the sample in the ice bath, avoid ultrasonic bubbles .

3.2 Re-clear samples, and IP overnight

3.2.1. After ultrasonic breaking, centrifuge at 12000rpm, 4℃ for 10min. Remove supernatant into a fresh tube. Transfer 90μL lysis solution for Input，store the others at -80 °C.

3.2.2. Take 40 μ l ultrasonic crushing product as input, add 10 μ L 5 * loading buffer, heat-denaturate it and perform WB detection to confirm that the sample contains the target protein. The remaining 50 μ l product was added with 2.5 μ l protease K and 2 μL 5M NaCl (the final concentration of NaCl was 0.2M), and the solution was decrosslinked at 55°C overnight;

3.2.3.Measure the nucleic acid concentration .Take part of the sample for PCR amplification, and then run agarose electrophoresis to detect the effect of ultrasonic ， and confirm whether the sample contains the target DNA;

3.2.4. After confirming the Input result, take 100 μl of the ultrasonically crushed product , add 900 μl of ChIP Dilution Buffer containing 1 mM-PMSF and 20 μl of 50×PIC (cooktail). Add 60 μl of Protein A+G Agarose/Salmon Sperm DNA for each sample. Incubate for 1 hours at 4ºC in a rotating wheel.

3.2.5. Incubate for 10 minutes at 4ºC ， centrifuge at 4000 rpm for 5 minutes;

3.2.6. Transfer the sample into two 1.5mL EP tubes.For the IgG tubes,add 1μg of IgG（of the same species）. For the IPs, use 2 μg of antibody per tube. Incubate overnight at 4ºC in a rotating wheel.

Day 2：

3.3 Wash samples, elute, and reverse crosslink.

3.3.1.Add 200μl Protein A+G Agarose/Salmon Sperm DNA to each tubes . Rotate for 2h at 4 °C to pull down immuno complexes.

3.3.2.Let all tubes standing at 4℃ for 10min.Centrifuge at 4000 rpm for 1 minutes. Discard the supernatant.

3.3.3.Wash the precipitate complex with the following solution in turn. Cleaning steps: add the solution, turn it upside down slightly, centrifuge at 4000rpm for 3min, and remove the supernatant.

Washing solution

1x low salt immune complex wash buffer.

1x high salt immune complex wash buffer.

1x LiCl immune complex wash buffer.

2x TE buffer.

The samples can be used for Western Blot analysis. Add 30µl 1X loading buffer to 30µl beads,then denature them for WB.

3.3.4.Freshly prepare elution buffer（0.5gSDS+0.42gNaHCO3+50ml Purified Water）. Add 200 µL of elusion buffer to Elute protein;

3.3.5.Add 20µl 5 M NaCl and 10µl proteinase K to the eluents. And heat all samples at 55 °C over night to reverse the crosslinking.

Day 3：

3.4 Purify DNA with Spin Column

3.4.1.Add 200 µl Buffer GB to the sample, mix thoroughly by vortex, and incubate at 70°C for 10min to yield a homogeneous solution. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the tube.

3.4.2.Add 200µl ethanol (96-100%) to the sample, and mix thoroughly by vortex for 15 s. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the tube.

3.4.3.Transfer the mixture from step 2 into the Spin Column CB3 (in a 2 ml collection tube) and centrifuge at 12,000 rpm(~13,400 × g) for 30s. Discard flow-through and place the spin column into the collection tube.

3.4.4.Add 500 µl Buffer GD(Ensure ethanol(96-100%) has been added) to Spin Column CB3, \ and centrifuge at 12,000 rpm(~13,400 × g) for 30s, then discard the flow-through and place the spin column into the collection tube

3.4.5.Add 700µl Buffer PW (Ensure ethanol (96-100%) has been added) to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g)for 30s. Discard the flow-through and place the spin column into the collection tube.

3.4.6.Add 500µl Buffer PW to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g)for 30s. Discard the flow-through and place the spin column into the collection tube.

3.4.7.Centrifuge at 12,000 rpm(~13,400 × g) for 2 min,and place it at room temperature for 10min to dry themembrane completely.

3.4.8.Transfer the Spin Column CB3 in a new clean 1.5 ml microcentrifuge tube, and add 50-200 µl Buffer TE directly to the center of themembrane. Incubate at room temperature(15-25°C) for 2min, and then centrifuge for 2min at 12,000 rpm(~13,400 × g)

3.5 DNA analysis（Real-Time PCR or DNA Sequencing）

3.5.1.Real-Time PCR

Using JASPAR or other database to predict the transcription factor binding sites. Design the primers according to the sites.

.Make a mixture in 200µl microcentrifuge tube

2× qPCR Mix 10µl

2.5μM primer	2μl
Template	2μl
ddH2O	6μl1

Run reaction

Pre-incubation	95℃，10min
Amplification: 40 cycles of	95℃，15s→60℃，60s
Melting curve	60℃→95℃，the temperature grows 0.3℃ every 15s

.Run PCR reaction on a 2.5% TAE gel and document.

3.5.2.DNA Sequencing

Collect DNA, measuring the concentration,and then send to a sequencing company.