Immunoprecipitation 实验报告

1、 实验器材

名称	厂家	型号
酶标仪	Rayto	RT-6100
研磨仪	Wanwu	KZ-II
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	Wanwu	BT-2
暗匣	Wanwu	WGA0017
暗室专用红灯	Wanwu	WGA0016
柯达胶片	Wanwu	WGJP0001
制冰机	SIMAG	SPR80
封口机	Wanwu	WGXYQ0004
图像分析软件	Adobe	Adobe PhotoShop
灰度分析软件	Alpha Innotech	alphaEaseFC

2、 主要实验试剂

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
PVDF 膜 0.45μm	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

3、 IP 实验步骤

（1）细胞蛋白提取

1.1 细胞培养（贴壁细胞：100mm 细胞培养皿中单层细胞长满 80%-90%或者细胞培养瓶中细胞达到 2-5×10^7 个，悬浮细胞：离心收集细胞，细胞达到 2-5×10^7 个）；

1.2 加适量预冷的 IP 细胞裂解液到细胞培养皿中（IP 裂解液中加入蛋白酶抑制剂），4℃裂解细胞 10min，期间用移液器反复吹打，然后将细胞悬浮液转移到 1.5ml 离心管中，继续冰上裂解 20min；

1.3 12000rpm、4℃离心 10min，将上清转入新的 1.5ml 离心管中，冰上操作，然后采用 BCA 法测定蛋白浓度；

1.4 取少量上清液变性后用于 input 实验，即 WB 检测靶蛋白。。

（2）组织蛋白提取

2.1 组织块用预冷的 PBS 洗涤 2-3 次，去除血污，剪成小块置于匀浆管中，加入 10 倍组织体积 IP 裂解液（使用前数分钟内加入蛋白酶抑制剂），机器匀浆。如果需要提高蛋白浓度，可以适量减少该 IP 裂解液体积；

2.2 将匀浆液转移至 1.5ml 离心管中，冰上裂解 30min；

2.3 12000rpm，4℃，离心 10min，收集上清，然后采用 BCA 法测定蛋白浓度；

2.4 取少量上清液变性后用于 input 实验，即 WB 检测靶蛋白。

（3）免疫沉淀步骤

3.1 向阴性对照（IgG）组别蛋白上清液中加入 1.0μg IgG（与 IP 实验用于沉淀的抗体种属来源相同的普通 IgG）和 20μL protein A/G 珠子（使用前充分混匀）,实验组别直接加入 20μL protein A/G 珠子，4℃摇转孵育 1h；

3.2 2000g，4℃，离心 5min，取上清；

3.4 加入 1-10μL（0.2-2μg）抗体，然后 4℃孵育过夜；

3.5 加入 80μL protein A/G-珠子（使用前充分混匀），用手指轻轻弹匀，4℃孵育 2h；

3.6 4℃，2000g，离心 5min，小心吸去上清，注意不要吸到底部的珠子，收集免疫沉淀复合物；

3.7 将免疫沉淀复合物用 1ml 预冷的 IP 裂解液（不用加各种抑制剂）洗涤 4 次，每次 4℃，2000g，离心 5min，每次洗涤小心弃去上清；

3.8 最后一次洗涤之后，尽可能的吸去上清液，然后,加入 80μL 1×还原型上样缓冲液，沸水煮 10min，4℃，1000g，离心 5min 取上清。

4、 检测

4.1 取 50μL 上清样品用于 SDS-PAGE 检测；

4.2 考马斯亮蓝染色后切胶回收蛋白，做质谱检测鉴定。

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
Table concentrator	Wanwu	TSY-B
Vertical Electrophoresis Apparatus	Wanwu	BV-2
Transfer Electrophoresis Apparatus	Wanwu	BT-2
Camera obscura	Wanwu	WGA0017
Darkroom lamp	Wanwu	WGA0016
Photographic film	Wanwu	WGJP0001
Ice Maker	SIMAG	SPR80
Sealer	Wanwu	WGXYQ0004
Image-analysis software	Adobe	Adobe PhotoShop
Gray analysis software	Alpha Innotech	alphaEaseFC

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
Phosphatase 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
PVDF membrane 0.45μm	Wanwu	G6015-0.45
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
Electrophoretic Buffer	Wanwu	G2018
TBS buffer solution	Wanwu	G0001

3、IP Protocol

（1） Preparation of lysate from cells

1.1 Cell culture（Adherent cells：The cells should be about 80% - 90% confluentin a 100 mm cell culture dish,or the number of cells in the culture bottle was2-5×10^7 ， Suspension cells：Centrifuge to collect cells（2-5×10^7）

1.2 Add enough cold IP buffer.（Protease and phosphatase inhibitors should be added before use），vibrate. Maintain constant agitation for 10 min in ice bath, use pipette to mix it up. Then gently transfer the cell suspension into a 1.5ml microcentrifuge tube. Continue splitting for 20 min in ice bath.

1.3 Centrifuge at 12,000 rpm, 4°C for 10 min. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice； then determination with the BCA kit

1.4 Denature some lysis solution for Input

（2） Tissue protein extraction

2.1 Wash the sample with cold PBS for twice or three times， then homogenize with an electric homogenizer. Volumes of lysis buffer must be determined in relation to the amount of tissue present.

2.2 Gently transfer the cell suspension into a 1.5ml microcentrifuge tube.Maintain constant agitation for 30 min in ice bath, use pipette to mix it up.

2.3 Centrifuge at 12,000 rpm ,4°C for 10 min. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice； Use BCA method to determine protein concentration.

2.4 Denature some lysis solution for Input.

（3） Protocol For IP

3.1 Add 1.0μg IgG and 20μL Protein A/G PLUS-agarose to the IgG group.Add 20μl Protein A/G PLUS-agarose to the experimental group. Shaken at 4 ℃ for 1 h.

3.2 Centrifuge at 2000g ,4℃ for 5 min. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant

3.3 Incubate 1-10μL（0.2-2μg） antibody coupled beads in 250 μl cell lysis supernatants, rotate overnight at 4 ℃

3.4 Mix the lysates with 80 μl Protein A/G PLUS-agarose rotating at 4 ℃ for 2 h.

3.5 Centrifuge at 2000g, 4℃ for 5 min. gently remove the supernatant.

3.6 Wash the immunoprecipitation complex with 1 ml of precooled IP lysate (without any inhibitors) for 4 times. Centrifuge at 2000g ,4℃ for 10 min , and the supernatant should be carefully discarded each time

3.7 After the last washing ,try best to remove the supernatant. Elute protein with 80μl 1x SDS protein gel sample loading buffer, boil for 10 min in boiling water. Centrifuge at 1000 g, 4 ℃, for 5 min. then aspirate the supernatant.

4、 Detection

4.1 Load 50μl protein sample into the wells of the SDS-PAGE gel, along with molecular weight marker.

4.2 Determine those sample by the dying method with coomassie brilliant blue. Extraction of protein from gel for mass spectrometry