Western Blot 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
裁纸刀	得力	NO.8014
超声波细胞破碎仪	宁波新芝生物	JY92-11N
通风橱	DGYUCH	DZ47-63C32
扫描仪	EPSON	V370
灰度分析软件	Alpha Innotech	alphaEaseFC
图像分析软件	Adobe	Adobe PhotoShop
化学发光仪	CLINX	6300
3mm 研磨珠	Wanwu	G0203-150G

2、 主要实验试剂

试剂	厂家	货号
RIPA 裂解液	Wanwu	G2002
50*cooktail	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*蛋白还原型上样缓冲液	Wanwu	G2013
SDS-PAGE 凝胶制备试剂盒	Wanwu	G2003
蛋白 Marker	Wanwu	26617
PVDF 膜 0.45um	Wanwu	G6015-0.45
脱脂奶粉	Wanwu	G5002
TWEEN 20	Wanwu	WGT8220
ECL	Wanwu	G2014
显影定影试剂	Wanwu	G2019
β-actin	Wanwu	GB12001
GAPDH	Wanwu	GB12002
Histone H3	Wanwu	GB11026
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

3、 Western Blot 实验步骤

（1）细胞总蛋白提取

1.1 对于悬浮细胞: 3000rpm，4℃，4 min，离心收集细胞沉淀，每 10^6 细胞加 250 μL 左右 RIPA 裂解液（在使用前数分钟内加入蛋白酶抑制剂），振荡。如果需要提高蛋白浓度，可适当减少裂解液使用量。

1.2 对于贴壁细胞:

1.2.1 用 PBS 冲洗细胞 2-3 次，最后一次清洗完，倒掉 PBS ，用移液器尽量吸干残留液体；

1.2.2 加入适当体积的 RIPA 裂解液（使用前数分钟内加入蛋白酶抑制剂）于培养板/培养瓶内 3-5min。期间反复晃动培养板/瓶，使试剂与细胞充分接触；

1.2.3 用细胞刮刀将细胞刮下，转移到 1.5ml 离心管中；

1.3 冰上裂解 30min，期间用移液器反复吹打，确保细胞完全裂解。

1.4 12000rpm,4℃，离心 10min，收集上清，即为总蛋白溶液。

（2）组织总蛋白提取：

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 1~2 个

3mm 的匀浆珠，加入 10 倍组织体积的裂解液（使用前数分钟内加入蛋白酶抑制剂），设置匀浆程序进行匀浆；如果需要提高蛋白浓度，可以适量减少裂解液体积；

2.2 将匀浆完成的匀浆管取出，放置冰上裂解液 30min，每隔 5min 震荡一次确保组织完全裂解；

2.3 12000rpm，4℃，离心 10min，收集上清，即为总蛋白溶液。

（3）**浆蛋白核蛋白提取**（细胞核蛋白与细胞浆蛋白抽提试剂盒） 准备溶液：室温解冻试剂盒中的三种试剂，解冻后立即放置在冰上，混匀。取适当量的细胞浆蛋白抽提试剂 A 备用，在使用前数分钟内加入 PMSF，使 PMSF 的最终浓度为 1mM。取适当量的细胞核蛋白抽提试剂备用，在使用前数分钟内加入 PMSF，使 PMSF 的最终浓度为 1mM；

3.2 对于**贴壁细胞**：用 PBS 洗一遍，用细胞刮刀刮下细胞，或用 EDTA 溶液处理细胞使细胞不再贴壁很紧，并用移液器吹打下细胞。离心收集细胞，尽最大努力吸尽上清，留下细胞沉淀备用。尽量避免用胰酶消化细胞，以免胰酶降解需抽提的目的蛋白；

3.3 对于**悬浮细胞**：用 PBS 洗一遍，离心收集细胞，尽最大努力吸尽上清，留下细胞沉淀备用。

3.4 对于**新鲜组织：**把组织尽可能切成非常细小的碎片。按照 20:1 的比例混合适当量的细胞浆蛋白抽提试剂 A 和 B(例如 200μL 细胞浆蛋白抽提试剂 A 中加入 10μL 抽提试剂 B),并加入 PMSF 至最终浓度为 1mM 配制成组织匀浆液。按照每 60mg 组织加入 200μL 裂解液的比例，加入裂解液，进行机器匀浆，匀浆需在冰浴或 4℃进行。冰浴放置 15min。4℃, 2000g 离心 5min，把上清转移至一预冷的 EP 管中，为抽提得到的部分细胞浆蛋白。(吸上清时千万不要触及沉淀。)到了这一步仍有部分浆蛋白未抽提出来，剩余沉淀按细胞沉淀的方法再次抽提浆蛋白；

3.5 每 20μL 细胞沉淀加入 200μL 添加了 PMSF 的细胞浆蛋白抽提试剂 A；

3.6 最高速剧烈涡旋 5s，把细胞沉淀完全悬浮并分散开；(如果细胞沉淀没有完全悬浮并分散开，可以适当延长涡旋时间。)

3.7 冰浴 10-15min；

3.8 加入细胞浆蛋白抽提试剂 B 10μL。最高速剧烈涡旋 5s，冰浴 1min；

3.9 最高速剧烈涡旋 5s，4℃ 12,000-16,000g 离心 5min；

3.10 立即吸取上清至一预冷的 EP 管中，即为抽提得到的细胞浆蛋白。可以立即使用，也可以冻存；

3.11 对于沉淀，完全吸尽残余的上清，加入 50μL 添加了 PMSF 的细胞核蛋白抽提试剂；

3.12 最高速剧烈涡旋 15-30s，把细胞沉淀完全悬浮并分散开。然后放回冰浴中，每隔 1-2min 再高速剧烈涡旋 15-30s，共 30min；

3.13 4℃ 12,000-16,000g 离心 10min；

3.14 立即吸取上清至一预冷的 EP 管中，即为抽提得到的细胞核蛋白。可以立即使用，也可以-80℃冻存；

（4）**蛋白浓度测定**：如果需要测蛋白浓度，取未变性的蛋白溶液，用 BCA 蛋白浓度测定

试剂盒测蛋白浓度，具体方法参照试剂盒说明书；

（5）**蛋白变性**：将蛋白溶液按照 4:1 的比例加入 5*还原型蛋白上样缓冲液，沸水浴变性 15min，收入-20°C冰箱保存备用；

（6）SDS-PAGE 电泳

6.1 清洗玻璃板；

6.2 制胶与上样；

6.2.1 待玻璃板自然晾干后，将一块凹玻璃板和一块平玻璃组成一对，放入制胶器，插入斜插板将玻璃板固定，检查底部是否对齐，避免漏胶；

6.2.2 根据实验需求配制不同浓度的分离胶，加入 TEMED 后立即混合均匀，将分离胶灌到适当的的高度，灌胶之前可以用梳子试一下，梳子齿距离分离胶上液面大约 5-8mm 为宜。然后将纯水缓慢均匀加入到分离胶上层，直至加满。大约 30min 后待分离胶凝固即可倒去分离胶上层水并用吸水纸将剩余水吸干；

	分离胶配比					
试剂	8%	10%	12%	15%	18%	20%
H_2O ml	4.63	4	3.3	2.3	1.3	0.63
30%丙烯酰胺（29：1） ml	2.67	3.3	4	5	6	6.67
1.5M TRIS－Hcl(PH 8.8) ml	2.5	2.5	2.5	2.5	2.5	2.5
10%SDS ml	0.1	0.1	0.1	0.1	0.1	0.1
AP ml	0.1	0.1	0.1	0.1	0.1	0.1
TEMED ul	5ul	5ul	5ul	5ul	5ul	5ul
总体积 ml	10ml					

试剂	**5%浓缩胶配比**
H_2O ml	2
30%丙烯酰胺（29：1）ml	0.5
1M TRIS－Hcl(PH 6.8)ml	0.5
10%SDS ul	40
AP ul	30
TEMED ul	4ul
总体积 ml	3ml

6.2.3 按照上述配方配制 5%的浓缩胶，加入 TEMED 后立即混合均匀，即可灌胶。将剩余空间灌满浓缩胶然后将梳子插入浓缩胶中，注意梳子下面不能有气泡。

6.2.4 等浓缩胶凝固好，取下制胶器，小心拔掉梳子，即可准备开始电泳；

6.3 将制胶器放入电泳槽后加足够的电泳液后上样电泳。浓缩胶电压 75V，分离胶用

120V。电泳至溴酚蓝里最底部大约 1cm 即可终止电泳，进行转膜。

（7）转膜

7.1 准备 6 张 7×9cm 的滤纸和一张大小适中的 PVDF（0.45um）膜，PVDF 膜在使用之前要先用甲醇活化 2min；

7.2 在加有转移液的盆里放入转膜用的夹子，两块海绵垫，一支玻璃棒，滤纸和经过活化的 PVDF 膜；

7.3 将夹子打开，左边白色，右边为黑色，在两边各加一块海绵、三层滤纸。

7.4 小心剥下分离胶放在滤纸上，将 PVDF 膜置于胶上，不要有气泡，在膜上盖三张滤纸并除去气泡。最后盖上另一个海绵垫；

7.5 转膜条件（湿转）**:**300mA 恒流转膜半小时，转膜过程中将转膜设备放在冰水中降温。

（8）免疫反应

8.1 将转印完的膜放入装有 TBST 的孵育槽中，快速涮洗一次，然后加上的脱脂牛奶，放置脱色摇床上，室温下封闭 30min；

8.2 按照抗体说明书，进行一抗稀释，配置好后，倒掉孵育槽中的封闭液，加入配置好的一抗，4℃孵育摇床过夜（摇床慢摇）；

8.3 回收一抗，用 TBST 快速涮洗膜三次，然后加入 TBST，放置脱色摇床上快速洗脱，每次 5min，洗三次；

8.4 将二抗用 TBST 按照 1:5000 的比例进行稀释，然后加入孵育槽中，放置摇床上慢摇，室温下孵育 30min；

8.5 用 TBST 快速涮洗膜三次，然后再加入 TBST，放置脱色摇床上快速洗脱，每次 5min，洗三次。

（9）化学发光 在暗室中将 ECL A 液和 ECL B 液两种试剂在离心管中等体积混合，在曝光匣上贴双层 PE 手套或者其他透明薄膜，将 PVDF 膜的蛋白面朝上放在曝光匣两层薄膜之间，加入混合好的 ECL 溶液充分反应，1-2min 后，去尽残液，盖上层薄膜开始压胶片。压完的胶片用显影、定影试剂进行显影和定影。根据不同的发光强度调整曝光条件。

（10）凝胶图像分析 将胶片进行扫描存档，PhotoShop 整理去色，Alpha 软件处理系统分析目标带的光密度值。

4、 WB 结果及分析（详见 Excel 灰度分析表格）

Western Blot protocol

1、Experiment instruments

Equipment	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
Vertical Electrophoresis apparatus	Wanwu	BV-2
Transfer electrophoresis apparatus	Wanwu	BT-2
Camera obscura	Wanwu	WGA0017
Red light	Wanwu	WGA0016
Kodak film	Wanwu	WGJP0001
Ice machine	SIMAG	SPR80
Capper	Wanwu	WGXYQ0004
Paper cutter	Deli	NO.8014
Ultrasonic breaker	Ningbo New zhi biology	JY92-11N
Fume cupboard	DGYUCH	DZ47-63C32
Scanner	EPSON	V370
Gray analysis software	Alpha Innotech	alphaEaseFC
Image analysis software	Adobe	Adobe PhotoShop
Chemiluminescence apparatus	CLINX	6300
3mm Mill bead	Wanwu	G0203-150G

2、Reagents

Reagents	Company	Catlog
RIPA buffer	Wanwu	G2002
50* cooktail	Wanwu	G2006
PMSF（100mM）	Wanwu	G2008
Phosphatase inhibitor	Wanwu	G2007
BCA protein quantitative detection kit	Wanwu	G2026
5*loading buffer	Wanwu	G2013
SDS-PAGE kits	Wanwu	G2003

Protein Marker	Thermo Fermentas）	26617
PVDF membrane 0.45μm	（Wanwu	G6015-0.45
Skim Milk	Wanwu	G5002
TWEEN 20	Solarbio	T8220
ECL	Wanwu	G2014
Developer and fixer	Wanwu	G2019
β-actin	Wanwu	GB13001-1
GAPDH	Wanwu	GB13002-m-1
Histone H3	Wanwu	GB11026
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	G0001-2L

3、 Western Blot Protocol

（1） Sample Preparation (Total protein)

1.1 For suspension cells: Centrifuge at 3000rpm，4°C for 4minutes to collect cells, add RIPA buffer. (250μL RIPA buffer/10^6 cells（Protease and phosphatase inhibitors should be added before use）. If you wish to increase protein concentration，then add less lysis buffer.

1.2 For adherent cells

1.2.1 Place the cell culture dish on ice and wash the cells three times with ice-cold PBS.

1.2.2 Aspirate the PBS, then add ice-cold RIPA buffer (Protease and phosphatase inhibitors should be added before use).

1.2.3 Scrape adherent cells off the dish，then gently transfer the cell suspension into a 1.5mL microcentrifuge tube.

1.3 Maintain constant agitation for 30 min in ice bath, use pipette to mix it up.

1.4 Centrifuge at 12,000 rpm，4°C for 10 min. Gently remove the tubes from the centrifuge and place on ice,tranfer the supernatant to a fresh tube.

（2） Preparation of lysate from tissues

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.

2.2 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 aspirate the supernatant and place in a fresh tube kept on ice.

（3）**Histone extraction protocol for western blot**（Use Nuclear protein and cytoplasmic protein extraction kit）

3.1 Prepare the reagents： Dissolve the reagents at room temperature, then mix them up and put them on ice. The Nuclear protein and cytoplasmic protein extraction reagent A and B should be mixed up with PMSF until its final concentration comes to 1mM. **Adherent cells**： Wash the cells three times with ice-cold PBS or EDTA. Centrifuge and collect the cells, discard the supernatant.

3.3 **Suspension cells**： Wash the cells three times with ice-cold PBS or EDTA. Centrifuge and collect the cells, discard the supernatant.

3.4 **Fresh Tissue**： Cut the tissue into very small pieces as much as possible. Mix the appropriate amount of cytoplasmic protein extract A and B at a ratio of 20:1 (e.g., add 10 microliter of cytoplasmic protein extract B to 200 microliter of cytoplasmic protein extract A). Prepare a tissue homogenate with a final concentration of PMSF of 1 mM. Add 200μL of lysis buffer per 60mg tissue for machine homogenization. The homogenization should be performed in an ice bath or 4°C..Place in an ice bath for 15 minutes. After centrifugation at 2000g , 4℃ for 5 minutes, the supernatant was transferred to a precooled EP tube, which was part of the cytoplasmic protein extracted.(Never touch the sediment when supernatant.)At this stage, some plasma proteins were still not extracted, and the remaining precipitation was again extracted by the method of cell precipitation.

3.5 Add 200μL of cytoplasmic protein extraction reagent A with PMSF into each 20μL of cell precipitate.

3.6 Vortex at the highest speed for 5s, let the cell precipitates completely suspended and dispersed;(Vortex time can be extended if cell precipitation were not completely suspended and dispersed).

3.7 Maintain constant agitation for 10-15 min in ice bath.

3.8 Add 10μL Cytoplasmic protein extraction reagent B(add PMSF before use).Vortex at the highest speed for 1s. Maintain constant agitation for 1 min in ice bath.

3.9 Vortex at the highest speed for 5s， Centrifuge at 12,000-16,000g, 4°C. for 5 min.

3.10 Aspirate the supernatant(plasma protein) and place in a fresh tube kept on ice.

3.11Aspirate the supernatant. Add 50μL nuclear protein extraction reagent B(add PMSF before use).

3.12 Vortex at the highest speed for 15-30s. Maintain constant agitation in ice bath. Vortex at the highest speed for 15-30s in between 1-2 minutes.(Total:30min).

3.13 Centrifuge at 12,000-16,000g, 4°C for 10 min.

3.14 Tranfer the supernatant to a fresh tube kept on ice. The supernatant is nuclear protein, you can immediately use or keep in -80℃ fridge.

（4）**Determination :** If the protein concentration needs to be measured, take some undenatured protein solution and measured by the BCA protein concentration measurement kit. The specific method is referred to the kit instructions

（5）**Denature the protein:** Add 5* reduced protein loading buffer to the protein solution at a ratio of 4:1, denature it in a boiling water bath for 15 minutes, and stored at -20℃ for later use

（6）**SDS-PAGE**

6.1 Washing glass plates.

6.2 Preparing and loading the gel.

6.2.1 After the glass panel naturally dried, a concave glass panel and a flat glass panel shall be formed into a pair, which shall be put into the gel-making device, inserted into the inclined glass panel to fix the glass panel, and check whether the bottom is aligned to avoid gel leakage;

6.2.2 Different concentrations of separation gel were prepared according to the experimental requirements, and mixed evenly immediately after adding TEMED, and the separation gel was poured to an appropriate height. A comb could be used to test the height before pouring, and the comb teeth should be about 5-8mm away from the liquid level of separation gel. Then add the pure water to the top layer of the separation gel slowly and evenly until it is filled .After about 30min, pour the water from the upper layer of the separation glue and drain the remaining water with absorbent paper.

separation gel buffer

	concentration											
Reagents	8%	10%	12%	15%	18%	20%	8%	10%	12%	15%	18%	20%
H_2O mL	4.63	4	3.3	2.3	1.3	0.63	6.9	5.9	4.9	3.4	1.9	0.9
30% acrylamide （29：1） mL	2.67	3.3	4	5	6	6.67	4	5	6	7.5	9	10
1.5M TRIS－HCl(PH 8.8) mL	2.5	2.5	2.5	2.5	2.5	2.5	3.8	3.8	3.8	3.8	3.8	3.8
10%SDS mL	0.1	0.1	0.1	0.1	0.1	0.1	0.15	0.15	0.15	0.15	0.15	0.15
AP mL	0.1	0.1	0.1	0.1	0.1	0.1	0.15	0.15	0.15	0.15	0.15	0.15
TEMED μL	5	5	5	5	5		7.5	7.5	7.5	7.5	7.5	7.5
Total	10 mL						15 mL					

5% stacking gel buffe**r**

Reagents	concentration 5%			
H_2O mL	2	3	4	6
30% acrylamide（29：1） mL	0.5	0.75	1	1.5
1M TRIS－HCl(PH 6.8)mL	0.5	0.75	1	1.5
10%SDS μL	40	60	80	120
AP μL	30	45	60	90
TEMED μL	4	6	8	12
Total mL	3	4.5	6	9

6.2.3 Prepare 5% concentrated gel according to the above formula. After adding TEMED, mix well immediately, and then the gel can be filled. Fill the remaining space with the concentrate and insert the comb into the concentrate. Make sure there are no bubbles under the comb.

6.2.4 After stacking gel has polymerized, remove comb carefully and begin the electrophoresis .

6.3 Add enough electrophoresis solution for sample electrophoresis., Concentrated gel voltage is 75V, and separation gel uses 120V. The electrophoresis was stopped when the bottom of bromophenol blue was about 1cm.

（7）Transferring the protein from the gel to the membrane

7.1 Prepare 6 pieces of 7×9cm filter paper and a PVDF (0.45um) membrane of moderate size. The PVDF membrane should be activated by methanol for 2min before use

7.2 In the basin with transfer buffer, place the clip for transfer film, two sponge pads, a glass rod, filter paper and activated PVDF film;

7.3 Open the clip with white on the left and black on the right. Add a sponge and three layers of filter paper on each side.

7.4 Carefully peel off the separation glue and put it on the filter paper. Put the PVDF film on the filter paper. Cover three pieces of filter paper on the film and remove the bubbles. Finally, cover with another spong.

7.5 **Transfer condition (**wet transfer) 300mA for 3omintues ,during the transfer process, the film transfer equipment is placed in ice water to cool down

（8）Antibody staining

8.1 Put the transferred membrane into the TBST incubation tank for a quick Wash, Block the membrane for 30minutes at room temperature by blocking buffer (5% milk,).

8.2 Incubate the membrane with appropriate dilutions of primary antibody. We recommend overnight incubation at 4°C.

8.3 Recycle the primary antibody then wash the membrane for three washes with TBST, 5 min each .

8.4 Incubate the membrane with the recommended dilution (1:5000) of conjugated secondary antibody in blocking buffer at room temperature for 30min.

8.5 Wash the film three times with TBST, 5minutes each time.

（9）**Chemiluminescence** Acquire image using darkroom development techniques for chemiluminescence, Perform ECL as described by the manufacturer, add ECL reagents for 1-2min at RT and capture WB image using various durations of exposure.

（10）**Image analysis** Scan the film, Organize and desaturate by using Photoshop. Use Alpha processing system to analyze the optical density value of the target band.

4、WB result and analysis（shown in Excel）