Western Blot 实验报告

1 实验原理

免疫印迹，又被称为蛋白质印迹（Western blot，WB），是一种复合性的免疫学检测技术。该方法通过利用 SDS-PAGE 技术，在生物样本中将蛋白质分子根据其分子量在凝胶上进行分离，随后通过电转移的方式将这些蛋白质转移到固相膜上。固相膜上的蛋白质充当抗原，与相应的抗体发生免疫反应，接着与酶标记的第二抗体发生反应。最终，通过底物显色或荧光成像等手段，检测电泳分离的特异性目的基因表达的蛋白质。

2 实验器材及试剂

2.1 实验器材

名称	厂家	型号
电子天平	英衡电子天平	YHB3003
酶标检测仪	MolecμLar Devices	SpectraMax M2
冷冻离心机	Haier Biomedica	LX-165T2R
纯水仪	芷昂仪器（上海）有限公司	Clever-S15
磁力搅拌器	Servicebio	MS-150
脱色摇床	Servicebio	DS-S 100
电泳仪	北京东方瑞丽	DYY-600C
化学发光成像系统	上海勤翔科学仪器有限公司	ChemiScope 6100
-80℃冰箱	Haier	BCD-501WDGR
电转仪	ACE Biotechnology	S-TRANS FW606

电泳槽	北京东方瑞丽	DYC-ZY2
匀浆仪	Servicebio	KZ-II
超声仪	宁波新芝生物有限公司	JY92-IIN

2.2 主要实验试剂

试剂	厂家	货号
RIPA 裂解液	杭州浩克生物	HKW2011
50*cooktail	杭州浩克生物	HKW2016
PMSF（100mM）	杭州浩克生物	HKW2017
磷酸化蛋白酶抑制剂	杭州浩克生物	HKW2018
BCA 蛋白定量检测试剂盒	杭州浩克生物	HKW2019
5*蛋白上样缓冲液	杭州浩克生物	HKW2020
SDS-PAGE 凝胶制备试剂盒	杭州浩克生物	HKW2028
蛋白质常规分子量标记(10-180kDa)	杭州浩克生物	HKW2024
PVDF 膜 0.45 μm	millipore	IPVH00010
PVDF 膜 0.22 μm	millipore	ISEQ00010
BSA 牛血清白蛋白	杭州浩克生物	HKW2084
TWEEN 20	Solarbio	T8220
超敏、高敏 ECL 化学发光试剂盒二合一	杭州浩克生物	HKW2095
β-actin	Proteintech	66009-1-Ig
α-Tublin	Proteintech	66031-1-Ig
GAPDH	Proteintech	60004-1-Ig
HRP 标记山羊抗兔	Proteintech	SA00001-2
HRP 标记山羊抗小鼠	Proteintech	SA00001-1
转膜缓冲液（干粉）	杭州浩克生物	HK2017

试剂	厂家	货号
电泳缓冲液（干粉）	杭州浩克生物	HK2018
TBS-T（干粉）	杭州浩克生物	HK0003

3 实验方法

3.1 细胞总蛋白提取

3.1.1 对于悬浮细胞:

离心收集细胞，每 10^6 细胞加 200 μL RIPA（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，可以适当减少细胞总蛋白提取试剂体积。

2.1.2 对于贴壁细胞:

1) 用 PBS 冲洗细胞 2-3 次。最后一次彻底吸干残留液。
2) 加入适当体积的 RIPA（使用前数分钟内加入蛋白酶抑制剂）于培养板、瓶内 3-5min。期间反复晃动培养板、瓶，使试剂与细胞充分接触。
3) 用细胞刮刀将细胞及试剂刮下，收集到 1.5mL 离心管中。
4) 冰浴 30min，期间用移液器反复吹打，确保细胞完全裂解。
5) 12000g 离心 5min，收集上清，即为总蛋白溶液。

3.2 组织总蛋白提取：

1) 组织块用冷 PBS 洗涤 2-3 次，去除血污，用吸水纸吸干，剪成小块置于研磨管中，加入一颗 5mm 的钢珠，将 EP 管置于液氮中 2min，取出 EP 置于匀浆器中研磨（65HZ，10s 工作，15s 停顿，-40℃）。
2) 当 EP 管中组织成粉末状，加入 10 倍组织体积本试剂（使用前数分钟内加入蛋白酶抑制剂）置于匀浆器中研磨（65HZ，工作 10s，停顿 15s，运行 20 次，-40℃）。
3) 将匀浆液转移至 1.5mL 离心管中，冰浴超声（200w，15%功率，超声 3s，停顿 5s，运行 3min）。

4) 12000g 离心 10min，收集上清，即为总蛋白溶液。

3.3 蛋白浓度测定：BCA 法测蛋白浓度。

3.4 蛋白变性：将蛋白溶液按照 4:1 的比例加入 5*上样缓冲液，金属浴 95℃变性 10min，收入-20℃或-80 冰箱保存备用；

3.5 SDS-PAGE 电泳

1) 将玻璃板对齐后放入夹中卡紧，操作时要使两玻璃对齐，以免漏胶。按实验安排配制分离胶，加入 TEMED 后立即摇匀即可灌胶。大约 45min 后可倒去胶上层水并用吸水纸将剩余水吸干。

10%分离胶配比

试剂	体积
H_2O	2 mL
30%丙烯酰胺（29：1）	1.65 mL
1.5M TRIS－Hcl（PH 8.8）	1.25 mL
10%SDS	50 μL
AP	50 μL
TEMED	5 μL

4%浓缩胶配比

试剂	体积
H_2O	1.05 mL
30%丙烯酰胺（29：1）	250 μL
1M TRIS-Hcl（PH 6.8）	188 μL
10%SDS	15 μL
AP	15 μL
TEMED	1.5 μL

2) 按上述方法配 4%的浓缩胶，加入 TEMED 后立即摇匀即可灌胶。将剩余空间灌满浓缩胶然后将梳子插入浓缩胶中。

3) 加足够的电泳液后上样电泳。将样品加入电泳孔中，电泳。浓缩胶电压 80V，分离胶用 120V。电泳至溴酚蓝刚跑出即可终止电泳，进行转膜。

3.6 转膜（小于 20kd 的蛋白使用 0.22μm 的 PVDF 膜。）

1) 电泳时将转印 PVDF 膜放入甲醇中活化 5min，再放入含有 S-TRANS 阳极缓冲液的孵育盒中平衡 5min。

2) 将完成电泳的凝胶放置在盛有去离子水的托盘中浸泡 2min。

3) 取两张 S-TRANS 高效转膜垫片放至含有 S-TRANS 阳极缓冲液的孵育盒中充分浸润将“三明治结构”（底部为阳极缓冲液浸润的垫片、上为转印膜、再上为胶、再上为阴极缓冲液浸润的垫片）放至转印盒中，盖好盒盖，盖销完全插入锁定槽后将转印盒滑入转印槽中，1.5A，10min。

4) 转印完成，取出转印膜。

3.7 免疫反应

1) 将转好的膜于室温下脱色摇床上用 5%的脱脂牛奶(0.5%TBST 配)，封闭 1h。

2) 稀释一抗（TBST 溶解，磷酸化蛋白使用 TBST 溶解的 5%BSA），4℃孵育过夜。

3) 用 TBST 在室温下脱色摇床上洗五次，每次 5min。

4) 将二抗用 TBST 稀释 10000 倍，室温下孵育 120min 后，用 TBST 在室温下脱色摇床上洗五次，每次 5min。

3.8 化学发光

将 ECL A 和 B 液按照 1:1 比例混合好后备用，将洗脱完的 PVDF 膜取出放在吸水纸上，稍微吸干膜上面的液体，将膜放入混合好的 ECL 发光液中，让液体完全浸

没膜，待反应 1min 之后，将膜取出放入化学发光仪托盘上，按照预设程序开始化学发光。

3.9 凝胶图像分析

PhotoShop 整理结果，Image J 软件处理系统分析目的条带的灰度值。

三、WB 结果及分析（具体详见 Excel 灰度分析表格）

Western Blot Report

1. Experimental principle

Western blot(WB), is a composite immunoassay technique. The method uses SDS-PAGE technology to separate protein molecules on a gel according to their molecular weight in a biological sample, and then transfer these proteins to a solid phase membrane by means of electrical transfer. The protein on the solid phase membrane acts as an antigen and reacts with the corresponding antibody, which then reacts with an enzyme-labeled second antibody. Finally, the proteins expressed by specific target genes isolated by electrophoresis are detected by means of substrate color development or fluorescence imaging.

2. Laboratory equipment and reagents

2.1 Laboratory equipment

Equipment	Manufacturers	Model
Electronic balance	Wuxi Ying Heng Electronics Co., Ltd	YHB3003
Full wavelength enzyme labeling instrument	MolecμLar Devices	Spectramax M2
High speed refrigerated centrifuge	Haier	LX-165T2R
Laboratory pure water system	Zhiang Instrument (Shanghai) Co., Ltd	Clever-S
Magnetic stirrer	Servicebio	MS-150
Decolorizing shaker	Servicebio	DS-S100

Vertical Electrophoresis apparatus	Beijing Dongfang Rayleigh Technology Co., LTD	DYY-600C
Chemiluminescence apparatus	Clinx	ChemiScope 6100
Ultra-low temperature refrigerator	Haier	DW-86L338J
semi-dry transfer unit	ACE Biotechnology	S-TRANS FW606
Electrophoresis tank	Beijing Dongfang Rayleigh Technology Co., LTD	DYC-ZY2
Tissue Grinder	Servicebio	KZ-II
Ultrasonic cell crusher	Scientz	JY92-IIN

2.2 Laboratory reagents

Reagents	**Manufacturers**	**Catlog**
RIPA buffer	Hangzhou Haoke Biotechnology Co., Ltd	HKW2011
50*cooktail	Hangzhou Haoke Biotechnology Co., Ltd	HKW2016
PMSF（100mM）	Hangzhou Haoke Biotechnology Co., Ltd	HKW2017
Phosphatase inhibitor	Hangzhou Haoke Biotechnology Co., Ltd	HKW2018
BCA protein quantitative detection kit	Hangzhou Haoke Biotechnology Co., Ltd	HKW2019
5*loading buffer	Hangzhou Haoke Biotechnology Co., Ltd	HKW2020
SDS-PAGE Gel preparation kit	Hangzhou Haoke Biotechnology Co., Ltd	HKW2028
Prestained Protein Marker(10-180kDa)	Hangzhou Haoke Biotechnology Co., Ltd	HKW2024
PVDF membrane 0.45 μm	millipore	IPVH00010

Reagents	Manufacturers	Catlog
PPVDF membrane 0.22 μm	millipore	ISEQ00010
BSA	Hangzhou Haoke Biotechnology Co., Ltd	HKW2084
TWEEN 20	Solarbio	T8220
ECL luminous liquid assembly	Hangzhou Haoke Biotechnology Co., Ltd	HKW2095
β-actin	Proteintech	66009-1-Ig
α-Tublin	Proteintech	66031-1-Ig
GAPDH	Proteintech	60004-1-Ig
HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L)	Proteintech	SA00001-2
HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L)	Proteintech	SA00001-1
Transmembrane buffer （Dry powder）	Hangzhou Haoke Biotechnology Co., Ltd	HK2017
Electrophoretic buffer （Dry powder）	Hangzhou Haoke Biotechnology Co., Ltd	HK2018
TBS-T（Dry powder）	Hangzhou Haoke Biotechnology Co., Ltd	HK0003

3. Experimental method

3.1 Extraction of total cell protein

3.1.1 For suspension cells:

Centrifuge the cells, add 200 μL RIPA per 10^6 cells (add a protease inhibitor within a few minutes before use), and oscillate. If the protein concentration needs to be increased, the volume of the total protein extraction reagent can be appropriately reduced.

3.1.2 For adherent cells:

1) Flush the cells with PBS 2-3 times. Drain the residue thoroughly one last time.
2) Add the appropriate volume of RIPA (add the protease inhibitor within a few minutes before use) in the culture plate and bottle for 3-5 minutes. During the period, the culture plate and bottle were repeatedly shaken to make the reagent fully contact with the cells.
3) Scrape off the cells and reagents with a cell scraper and collect them into a 1.5mL centrifuge tube.
4) Ice bath for 30min, during which the pipette is blown repeatedly to ensure complete cell lysis.
5) Centrifuge 12000g for 5min and collect the supernatant, which is the total protein solution.

3.2 Preparation of lysate from tissues：

1) The tissue blocks were washed with cold PBS for 2-3 times to remove the blood stain, dried with absorbent paper, cut into small pieces and placed in the grinding tube, adding a 5mm steel ball, placing the EP tube in liquid nitrogen for 2min, taking out the EP and placing it in the homogenizer for grinding (65HZ, 10s operation, 15s stop, -40℃).
2) When the EP tube is organized into powder, add 10 times the tissue volume of this reagent (add the protease inhibitor within a few minutes before use) and grind it in a homogenizer (65HZ, work for 10s, stop for 15s, run 20 times, -40℃).

3) Transfer the homogenate into 1.5mL centrifuge tube for ice bath ultrasound (200w, 15% power, ultrasound 3s, pause for 5s, run for 3min).

4) Centrifuge 12000g for 10min and collect the supernatant, which is the total protein solution.

3.3 Determination of protein concentration: Determination of protein concentration by BCA protein quantitative detection kit.

3.4 Denature the protein: Add 5* loading buffer to the protein solution at a ratio of 4:1, denature it in a Dry Bath Incubator at 95℃ for 10 minutes, then stored at -20℃ or -80℃ refrigerator for later use.

3.5 SDS-PAG Eelectrophoresis

1) Align the glass plate and put it into the clamp. Align the two glasses during operation to avoid leakage of glue. The separation glue was prepared according to the experimental arrangement. After adding TEMED, shake well immediately to fill the glue. After about 45 minutes, the water above the glue can be poured and the remaining water can be sucked up with absorbent paper.

The ratio of 10% separating glue

Component	Volume
H_2O	2 mL
30％ acrylamide（29：1）	1.65 mL
1.5M TRIS－Hcl（PH 8.8）	1.25 mL
10％SDS	50 μL
AP	50 μL
TEMED	5 μL

The ratio of 4% concentrated glue

Component	Volume
H_2O	1.05 mL
30％acrylamide（29：1）	250 μL
1M TRIS-Hcl（PH 6.8）	188 μL
10％SDS	15 μL
AP	15 μL
TEMED	1.5 μL

2) Mix 4% concentrated glue according to the previous method, add TEMED and shake well immediately to fill glue. Fill the remaining space with glue concentrate and insert the comb into the glue concentrate.

3) Add enough electrophoresis solution before loading the sample. The sample is added to the electrophoresis hole, electrophoresis. The concentrated glue voltage is 80V, and the separation glue is 120V. When the bromophenol blue has just run out, the electrophoresis can be terminated and the membrane can be transferred.

3.6 Transferring the protein from the gel to the membrane

1) During electrophoresis, the transferred PVDF membrane was activated in methanol for 5min, and then balanced in a incubator box containing S-TRANS anode buffer for 5min.

2) Place the gel that completes the electrophoresis in a tray filled with deionized water and soak for 2min.

3) Take two S-TRANS high-efficiency transfer gaskets and put them into the incubator box containing S-TRANS anode buffer to fully infiltrate. Put the "sandwich structure" (the gasket with anode buffer at the bottom, transfer film on top, glue on top, and cathode buffer on top) into the transfer box, and close the lid. Slide the transfer box into the transfer slot after the cancelling is fully inserted into the locking slot, 1.5A, 10min.

4) Transfer completed, remove the transfer film.

3.7 Antibody staining

1) The transformed film was closed for 1h with 5% skim milk (prepared with 0.5%TBST) on a decolorized shaker at room temperature.
2) Dilute primary antibody (TBST dissolved, phosphorylated protein using 5%BSA dissolved by TBST) and incubate overnight at 4℃.
3) Wash with TBST on the decolorizing shaker at room temperature five times, 5min each time.
4) The second antibody was diluted 10,000 times with TBST, incubated at room temperature for 120min, and washed five times with TBST on the decolorizing shaker at room temperature for 5min each time.

3.8 Chemiluminescence

Mix liquid ECL A and B in a 1:1 ratio and set aside. Take out the eluted PVDF membrane and put it on absorbent paper, slightly absorb the liquid above the membrane, put the membrane on the chemiluminescence meter shelf, add the mixed ECL luminescent liquid, let the liquid completely soak the membrane, after the reaction for 1min, use absorbent paper to absorb the excess liquid above, put it into the chemiluminescence meter, start chemiluminescence according to the preset procedure.

3.9 Analysis

Use PhotoShop software to sort out the results, and analyze the gray value of the target strip by Image J software.

4. Results (detailed in Excel gray analysis table)