EMSA 实验报告

1、 实验器材

名称	厂家	型号
酶标检测仪	Rayto	RT-6100c
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
超净工作台	苏净安泰	SW-CJ-1FD
暗室专用红灯	Wanwu	WGA0016
柯达胶片	Wanwu	WGJP0001
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

2、 主要实验试剂

试剂名	产地	货号
核蛋白抽提试剂盒	碧云天	P0028
EMSA 试剂盒	Thermo Fisher	20148
10*TBE	Wanwu	G3002
40％丙烯酰胺	Wanwu	G2005
50%甘油	Wanwu	G0120
10％AP	Wanwu	G2003-5

3、 EMSA 操作步骤

（1） 核蛋白提取（核蛋白与细胞浆蛋白抽提试剂盒）

1.1 准备溶液：室温融解试剂盒中的三种试剂，溶解后立即放置在冰上，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;

1.2 对于贴壁细胞：用 PBS 洗一遍，用细胞刮子刮下细胞，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；

1.3 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；

1.4 对于新鲜组织：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；

1.5 每 20μL 细胞沉淀加入 200μL 添加了 PMSF 的细胞浆蛋白抽提试剂 A。(对于二百万 Hela 细胞，其细胞沉淀的体积大约为 20μL 或 40mg。)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。) 冰浴 10-15min； 加入细胞浆蛋白抽提试剂 B 10μL。最高速剧烈涡旋 5s，冰浴 1min； 最高速剧烈涡旋 5s，4℃ 12,000-16,000g 离心 5min； 立即吸取上清至一预冷的塑料管中，即为抽提得到的细胞浆蛋白。可以立即使用，也可以冻存；(千万不要触及沉淀，可以在沉淀上方保留极小体积的上清，以免触及沉淀。)

1.11 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；(不吸尽上清会带来细胞浆蛋白的污染。) 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；4℃ 12,000-16,000g 离心 10min； 立即吸取上清至一预冷的塑料管中，即为抽提得到的细胞核蛋白。可以立即使用，也可以-80℃冻存；

（2）制胶 （1.5mm 胶，大约 30min 胶会凝）

试剂	浓度 5.5%
H_2O ml	7.50
10*TBE μl	500

40%丙烯酰胺 ml	1.50
50%甘油 μl	500
10%AP μl	50
TEMED μl	10
总体积 ml	10

待胶完全凝固后 120v 预电泳 1h，缓冲液用预冷的 0.5×TBE。

（3）蛋白与探针反应(注：蛋白浓度 1 μg/μl ,上样体积 2 μl)

试剂名称	阴性对照 μl	样品组μl	冷竞争μl	突变竞争μl	超迁移μl
10×binding buffer	2	2	2	2	2
1μg/μl poly（dl.dc）	1	1	1	1	1
50%glycerol	1	1	1	1	1
1%NP-40	1	1	1	1	1
100mM MgCl2	1	1	1	1	1
200mM EDTA	1	1	1	1	1
Protein	0	2	2	2	2
标记探针	2	2	2	2	2
未标记探针	0	0	2	0	0
突变探针	0	0	0	2	0
特异性抗体	0	0	0	0	2
DEPC 水	补足至 20μl				

混合后室温放置 25-30min。

（4）上样

预电泳完后马上更换预冷的电泳缓冲液，加 4μl 的 6×上样缓冲液到样品混合液中，马上上样电泳，150v ，60min。

（5）电转移

将带正电的尼龙膜放入 0.5×TBE 中平衡 10min。待电泳完成后将加有样品的整块胶取下电转。转膜缓冲液为预冷的 0.5×TBE，300mA 转印 30min。

（6）交联

转膜完成后将膜取出，于紫外灯下 20cm 处作用 20min。

（7）封闭

将交联完成的尼龙膜取出放入洗净的孵育槽，用封闭液封闭 20min。期间放于摇床上轻柔摇晃。(慢摇)。

（8）抗体反应

倒掉封闭液。用封闭液将抗体稀释 300 倍后，与膜完全作用 30min。期间放于摇床上轻柔摇晃。(慢摇).

（9）洗脱

用 1×的洗脱液清洗 4 次,每次 10min。摇床速度加大。

（10）ECL 发光检测：

将膜从洗脱液中取出，稍微吸干上面的液体，放入化学发光仪中，加上化学发光液，待反应 1-2min 后，吸尽多余的液体，开始化学发光。

4、图像分析（灰度比分析结果见 EXCEl 表格）

EMSA 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
Vertical Electrophoresis Apparatus	Wanwu	BV-2
Transfer Electrophoresis Apparatus	Wanwu	BT-2
Camera obscura	Wanwu	WGA0017
Bechtop	AIRTECH	SW-CJ-1FD
Darkroom lamp	Wanwu	WGA0016
Photographic film	Wanwu	WGJP0001
Image-analysis software	Adobe	Adobe PhotoShop
Gray analysis software	Alpha Innotech	alphaEaseFC
luminometer	CLINX	6300

2、 Reagents

Reagents	Company	Catlog
Nuclear protein and Cytoplasmic Protein Extraction Kit	Beyotime	P0028
EMSA kits	Thermo Fisher	20148
10*TBE	Wanwu	G3002
40％ Acrylamide	Wanwu	G2005
50% Glycerin	Wanwu	G0120
10％AP	Wanwu	G2003-5
TEMED	Wanwu	G2003-6

3、 Protocol For EMSA

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

1.1 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.

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

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

1.4 **From fresh tissues**： 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.

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

1.6 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).

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

1.8 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.

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

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

1.11Aspirate the supernatant. Add 50μL nuclear protein extraction reagent B(add PMSF before use).

1.12 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).

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

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

（2）Prepare 1.5mm native polyacrylamide gel and 0.5x TBE

Reagents	Concentration 5.5 %
H_2O ml	7.50
10*TBE μl	500
40％Acrylamide ml	1.50
50% Glycerin μl	500
10％AP μl	50
TEMED μl	10
Total ml	10

Pre-electrophoresis for 60 min at 120 V .Using precool 0.5×TBE as buffer solution.

（3）Prepare reaction mixtures as follows during pre-electrophoresis

Reagents	Negative Control μl	Sample μl	Competitor μl	Mutated competitorμl	Super Shift μl
10×binding buffer	2	2	2	2	2
1μg/μl poly（dI.dC）	1	1	1	1	1
50％glycerol	1	1	1	1	1
1%NP-40	1	1	1	1	1
100mM MgCl2	1	1	1	1	1
200mM EDTA	1	1	1	1	1
Protein	0	2	2	2	2
Labeled Probe	2	2	2	2	2
Competitor	0	0	2	0	0
Mutated competitor	0	0	0	2	0
antibody	0	0	0	0	2
DEPC	Add to 20μl				

Place at room temperature for 25-30 min。

（4）Load the sample

After pre-electrophoresis ,change precool running buffer. Add 4 μl of 6X DNA loading buffer to sample mixture. Load the samples on gel and run the gel at 150 V for 60min.

（5）Transfer

Put the positively charged nylon membrane into 0.5×TBE to equilibrate for 10 min. After the electrophoresis , remove the whole gel with the sample. The transferring condition is 300mA for 30min, using precool 0.5×TBE as buffer solution

（6）Crosslinking

Place the membrane containing the reaction mixture under the UV lamp (20 cm below the light) for 20min.

（7）Blocking

Block the membrane on a table concentrator for 20min at room temperature using blocking buffer.

（8）Antigen-Antibody Reaction

Aspirate the blocking buffer. Incubate the membrane with appropriate dilutions of primary antibody (1:300). for 30min at room temperature.

（9）Washing

Wash the membrane for 4 times(10min each) with 1X washing buffer.

（10）Chemiluminescence：

Take the membrane out of the eluent, absorb the liquid, put it in a chemiluminescence instrument, add ECL, wait for 1-2 minutes, absorb the excess liquid, and start chemiluminescence.

4、Result and analysis（shown in Excel）