**The protocols for J744 culture**

**1. make EDTA**

……weigh 0.05g EDTA,put into the bottle.

…… add 100ml distilled water to the bottle

……adjust the PH by NaoH to 7.4

……put the bottle to autocalve

……take out the bottle ,and tighten the cap.

**2.cell culture**

……discharge the medium

……wash cell with PBS for one time

……add 5 ml 0.05% EDTA (T-75),and put the flask into the incubator for 5min.

……take out flask from the incubator add 5ml medium to the falsk.

……transfer the mixture solution to 15ml centrifuge tube.

…… centrifuge at 1000rpm,5min

…… discharge the supernatant,resuspened the precipitation with 15 ml medium.and transfer to T-75.

**The protocols for MTS experiment**

…… get the cell like passage,and seed the cell with number 70,000/well at day 1.(100ul)

……add the 2× drug solution at day 2 .(100ul)

……dishcarge 100ul of the medium,and add 20ul MTS to the cell.

……put the plate into the incubator for 2 hour.

…… measure the OD at 490nm.

**The protocols for Translation and micoscope**

1.prepare the coverslips needed in the transfection:

a.put the coverslips in to the acid overnight.

b.wash the coverslips for 10 times with distilled water

c.put the coverslips in original ethyl alcohol

2.put the coverslips into the 6 well in the hood,open the windower in the hood to make the coverslips dry.after that open the UV for 20mins

**3.add 0.01%poly-lysin on the coverslips,at the same time you can prepare the cell like passage.**

**4.discharge the poly-lysin,and wash with pbs for three times.**

**5.seed the cell 3\*105 every well.**

**6.after 24h,do transfection,the plasmid 4ug with lipofectamin2000 2ul every well.**

a.prepare the tube

b.add 100ul medium without serum to two tube respectively.

c.one tube add plasmid,another one add lipofectamin2000.

d.mix the solution of the two tube,and wait for ,20min,before add to the cell.

**7.After24h,fix the cell**

a. wash the coverslip with the pbs 3 times

b.add the 4%PAF 20mins

c.wash the coverslip with the pbs 3 times

d.put the coverslips on the paper to make it dry

e.put the fix reagent a drop on the coverslip

f.put the coverslip with the cell side on it.

g.put those coverslips in the dark

**8. observe the cell.**

**The Protocols for WB**

**一、Protein Extraction**

**1.Whole protein: lysis buffer-A(100ml)**

50mM Tris-Hcl（PH8.8） 0.6057g

150mM Nacl 0.8766g

0.02%NaN3（Sodium azide ） 0.02g

0.1%SDS 0.1g

1%NP-40 1ml

0.5% sodium deoxycholate 0.5g

1mM EDTA 0.037224g

**2.Nuclear and cytoplasm protein**

**lysisi buffer –B(100ml)**

HEPES,PH7.9 10mM

Mgcl2 1.5mM

Kcl 10mM

**Lysis buffer-C(100ml)**

HEPES,PH7.9 20mM

Mgcl2 1.5mM

Nacl 0.42mM

EDTA 0.2mM

glycerol 25%（V/V）

**（一）Whole protein Extract**

1. Discharge the medium, centrifuge at 1000rpm,5min.

2.Add cold PBS, resuspened the precipitation, centrifuge at 1000rpm,5min.

3.Repeated step 2 again.

4.Add 35ul lysis buffer-A(every 1ml **lysis buffer-A** including PMSF 1Mm),cell lysis on ice for 15min.

**5.** Centrifuge at 12000rpm,30min,4℃

6.Get the supernatant(whole protein), and put into 1.5ml tube.

**（二）Nuclear and cytoplasm protein**

1. Discharge the medium, centrifuge at 1000rpm,5min.

2.Add cold PBS, resuspened the precipitation, centrifuge at 1000rpm,5min.

3. Repeated step 2 again.

4. Estimate cell volume,add 5 fold **lysisi buffer –B(**(every 500ul including 0.5ul 1M DTT 5ul and cocktail 5ul)to resuspened the precipitation

5.Incubation on ice for 15min.

6. Centrifuge at 10000rpm,2min,get the **cytoplasm protein.**

7. Add 70ul **extract buffer-c** (every 100ul extract buffer-c including 1ul 0.1M DTT and 1ul cocktail) resuspened the precipitation.

8. Incubation on ice for 15min.keeping vortex during this process.

9. Centrifuge at 14000rpm,5min,get the **nuclear protein.**

**二、BCA quantify.**

Do as the BCA Protocol.

**三、Protein sample prepare**

1.Accroding the protein concentration quantify ,add the 5🞨loading buffer, the load protein quantity is 25ug.

2.boil the sample at 100ºC for 10mins。

3.keep the sample for use.

**四、SDS-PAGE**

1.Prepare the gel as sharron suggest.

|  |  |
| --- | --- |
| 10%AP (Ammonium Persulfate) | 1L |
| AP | 0.1(g) |
| ddH2O | 100ml |

|  |  |
| --- | --- |
| 8% speration gel | 5ml |
| ddH2O | 2.295ml |
| 40%Acr-Bis | 1.25ml |
| 1M PH 8.8 Tris-Hcl | 1.3ml |
| 10% SDS | 50ul |
| 10%AP | 100ul |
| TEMDE | 5ul |

|  |  |
| --- | --- |
| 5% concentration gel | 5ml |
| ddH2O | 3.05ml |
| 40%Acr-Bis | 0.5ml |
| 1M PH 6.8 Tris-Hcl | 1.3ml |
| 10% SDS | 50ul |
| 10%AP | 100ul |
| TEMDE | 5ul |

2. When gel solidification,we put the 1🞨running buffer in the box,add the sample.

|  |  |
| --- | --- |
| 10🞨running buffer | 1L |
| Trisbase | 30.8(g) |
| Glycine | 188(g) |
| SDS | 10(g) |

3. SDS-PAGE gel condition

Separate gel:80V,30min

Concentration gel:120v ,1h20min.

**五、Trarsmembran**

1.put the membrane in [methanal](javascript:void(0);) for 20secs,then put the membrane in water for 2mins.

2.put the the membrane,gel,filter in pre-cold TBS for 20mins.

|  |  |
| --- | --- |
| 1🞨TB | 1L |
| Trisbase | 2.42(g) |
| Glycine | 11.52(g) |
| Methanol | 200ml |

3.transfer condition

300mA，2h

**六、Blocking**

After Trarsmembran,we get out of the membrane.put the membrane in the 5% BSA or milk for 2h. 10 mls

**七、Incubation primary antibody**

Use blocking solution（5%BSA）dilute the primary antibody (1:500),and incubate at 4 ºC overnight.

10 ml

**八、Washing membrane**

Use TBST wash the membrane by TBST 3 times, every time 10mins.

|  |  |
| --- | --- |
| 1🞨TBST | 1L |
| Trisbase | 6.04(g) |
| Nacl | 8.76(g) |
| 0.1%Trition-100 | 1ml |

**九、Incubation secondary antibody**

Use blocking solution（5%BSA）dilute the secondary antibody (1:500),and incubate at room temperature for 1.5h.

**十、Washing membrane**

Use TBST wash the membrane by TBST 3 times, every time 10mins.

**十一、ECL**

1. Put the membrane on the [plastic](javascript:void(0);) [wrap](javascript:void(0);)

2. put the enough HyGLO Quick SprayTM on the [plastic](javascript:void(0);) [wrap](javascript:void(0);),incubation 5mins in incubation.

3. [suction](javascript:void(0);) surplus HyGLO Quick SprayTM ,put the membrane in the black box,exposure for 1h(first time 10s,sum time 1h,30 pictures).

4.save the pictures.