Cytoprotein Extraction, Quantification & Western Blotting

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1 Introduction

The growth, development, and maintenance of multicelluar organisms depend only on the production of cells but also on mechanisms to destroy them. Normally, cell death is not random but under well control. In most of cases, **programmed cell death** occurs by a process called **apoptosis** — from the Greek word meaning "falling off", as leaves from a tree. A family of specialized intracellular protease trigger apoptosis. Typically, these proteases have a cysteine at their active site and cleave their target proteins at specific aseptic acids, which are therefore called **caspase**(c for cysteine and asp for aseptic acid). Pro-caspase-3 is a 32 kD proenzyme protein without activity. During apoptosis this protein is cleaved to p17 and p10 fragments and forms active caspase-3, the predominate terminal caspase in cell apoptosis.

We plan to detect activated caspases-3 in apoptosis by western blot in this experiment. We design this experiment to find out the cytoprotein **caspases** and **tubulin** between apoptosis cell and normal cell by western blotting. For western blotting, we procedure sample preparation, BCA protein assay, gel electrophoresis and membrane transfer and immunostainign steps.

Blotting refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane, which is a method to study cell biology. **Western blot** is kind of blotting which separates and characterize proteins by protein size and antibody-antigen interaction.

Firstly, we use lysis buffer to lysate cell from cell culture. To prepare to load sample into gel, we determine the protein concentration by the BCA Protein Assay. Lysis buffer containint protease and phosphates inhibitors, which disrupts the cell membrane and solubilizes intracelluar proteins. RIPA(radio immuniprecipitation assay) is used to study cytoprotein and membrane-bound protein, where we plane to detect tubulin and caspases-3 during apoptosis. After dealing with RIPA, samples should be added appropriate inhibitors(EDTA for this experiment) and keep at 4°C at all the time in order to slow down proteolysis, dephosphorylation and denaturation.

Not all the protein will be used to do western blotting, but we only load special weight of samples. We measure sample based on a standard curve using **BCA Protein Assay** as reference. Bicinchoninic acid(BCA) is the key component in this assay, which reacts with cuprous ions to generate an intense purple color at 562nm. the intensity of color is appropriately linear correlate to protein concentration.

Secondary, we do SDS-PAGE to separate proteins according to their molecular weight. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross-linked by methyl groups. The separation of molecules within a gel is determined by the relative size for the pores formed within the gel. To avoid the bias of different lane, the housekeeping genes(tubulin is used) are used as a reference.

Finally, we transfer proteins from gel to membrane, block the membrane, then incubate with the primary antibody and secondary antibody, detect the second antibody.

After SDS-PAGE, we can visualize the size and level of protein. More, we can detect more special protein using appropriate antibody. For this case, we transfer protein from a gel not stained by Coomassie brilliant blue to a new membrane. The principle is that, protein-SDS compounds with negative charges can induced to travel in an electrical field from a gel onto a sturdy support, a membrane that "blots" the protein from gel. Based on our target protein size, PVDF(polyvinylidene fluoride) membrane are usually be chosen, the pore of this membrane is 0.22 micron. PVDF membrane required carefully pre-treatment. Transfer can be done in wet or semi-dry conditions. The membrane should be packaged as sandwich structure(Figure 1) without air bubbles for wet transfer, while the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

These membranes are blocked by block buffer at 4°C overnight to prevents non-specific background bonding for the primary or secondary antibodies to the membrane. Then membranes incubate with primary antibody and secondary antibody. For detecting the protein-primary antibody-secondary antibody, we usually conjugate the second antibody with an enzyme. We use HRP-conjugate antibody to detect the change of color.

2 Materials & Method

2.1 Cytoprotein extraction

Centrifuge, water bath, CO_2 incubator, hood, orbital shaker, pipettes and tips, cuvette, forceps, tissue culture dish, 15mL centrifuge tube, 1.5 centrifuge tube, Pasteur pipette.

RPMI 1640 supplied with 10%FBS, FBS, staurosporine(1mM stock), Methanol, RIPA lysis buffer(Beyotime P0013B: 50mM tris(pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluorid, EDTA, leupeptin, etc. PMSF was mixed), Jurkat cell.

Jurkat cells was seeded to a dish, treat cells with 1μM staurosporine for 3 hr as experiment group and set a well not treated as control(Prepared by teacher). $\longrightarrow$ Harvest cell to 15mL tube $\longrightarrow$ centrifuge at 250xg, 5min $\longrightarrow$ Discard suspernatant, then add 200 μL RIPA lysis buffer and mix $\longrightarrow$ Incubate tube on ice for 30 min $\longrightarrow$ Transfer the cell lysate to 1.5mL tube $\longrightarrow$ Centrifuge 1300xg, 4°C $\longrightarrow$ Transfer suspernatant to new 1.5mL tube.

2.2 Cytoprotein quantification

Spectrophometer(Dynamica HALO XB-10), BCA protein quantification kit(Beyotime, P0009), PSA.

First, we made the standard curve of different concentration BSA solution as followin table. BCA solution should be added together in the end.

	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	Ctrl	Exp
PBS(5X)	100	95	90	85	80	75	70	65	95	95
0.5mg/mL BSA(μL)	0	5	10	15	20	25	30	35	0	0
sample(μL)	0	0	0	0	0	0	0	0	5	5
BCA work solution(μL)	900	900	900	900	900	900	900	900	900	900

Table 1. Sample for BSA standard curve.

Then, all the tube were incubated in 60°C water bath for 30min. We cooled down all sample to room tempeture and briefly spin down. All sample are transfer to clean cuvtte and measure the absorption at 562nm in spectrometer after using S_0 to blank. Form BSA absorption data, we got the standard curve and concentration $c(\text{ctrl}), c(\text{exp})$ of cytoprotein of control group and experiment group.

2.3 Western blotting

Precast SDS-PAGE gel(Bio-Rad), 3mm filfter paper, PVDF membrane(Millipore), Transfer Buffer(25mM Tris-HCl, pH8.3, 192mM glycine, 20%(v:v) methanol), SDS-PAGE running buffer(Tris 0.025M, Glycine 0.192M, SDS 0.1%, pH8.3), QuickBlock Western Blocking(Beyotime, P0252), Methanol, PageRuler$^{\text{TM}}$ Prestained Protein ladder(Thermo), Protein electrophoresis system(BIO-RAD), transfer system(BIO-RAD).

Mouse Anti-α-tubulin monoclonal antibody(BOSTER BM1452, diluted in QuickBlock Primary Antibodfy Dilution Buffer for Western Blot(Beyotime, P0256), 1:500), Rabbit Anti-CASP(p17) polyclonal antibody(BOSTER PB1083, diluted in Primary Antibodfy Dilution Buffer for Western Blot, 1:1000), HRP-goat-anti-mouse IgG monoclonal antibody(BOSTER, BM1050)(diluted in QuickBlock Secondary Antibody Dilution Buffer for Western Blot(Beyotime, P0258), 1:2000), HRP-goat anti rabbit IgG(BOSTER, BA1054, diluted in QuickBlock Secondary Antibody Dilution Buffer for Western Blot(Beyotime, P0258), 1:2000), Pierce$^{\text{TM}}$ ECL Western Blotting Substrate(Thermo Fisher, 32106)

First, 20μg(9.1μL) lysate of control group and experiment group were added to 1.5mL tube one by one, while RIPA was used to adjust final volume to 32μL. More, 8μL of 5X SDS-PAGE loading buffer was added to make final concentration 0.5μg/μL. These 2 sample were boiled at 100°C for 5min and centrifuge and store at -20°C.

Then 5 μL PageRuler$^{\text{TM}}$ Prestained Protein ladder, two 10 μL samples on a precast gel. We ran SDS-PAGE at 30V for 10min, then rise to 200V for 30min. SDS-PAGE gel is took out and made a sandwich as follows:

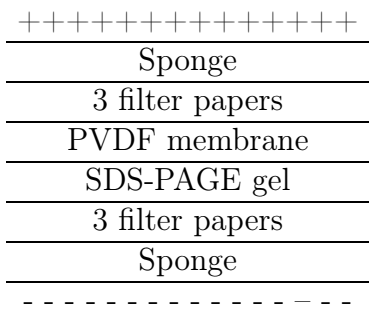

Figure 1. Sandwiched Structure

PVDF membrane was immersed to methanol for 1 min to active membrane before making sandwich. Setting up all parts and running 200mA for 2hr. Membrane is taken out, and was cut corner to indicate protein side. Membrane was washed once by TBST. We put this membrane to blocking buffer with protein upside, which are blocked at 4°C overnight with shaking. We used TBST to store blocked membrane for one week. Primary antiboby(1:400 diluted in TBST) was added incubate at RT for 1hr with shaking. Membrane was washed with TBST 3 times.

Finally, ECL substrate was added and taking image of membrane.

3 Results

1. The standard curve is

$$y = 0.03874x - 0.007$$

   where y is the absorption at 562nm, and x is the concentration of BSA solution(Figure 2).

2. The concentration of extraction cytoprotein are calculated as

	Abs at 562nm	Original Concentration
control group	0.446	$2.339 \mu g / \text{mL}$
experiment group	0.417	$2.189 \mu g / \text{mL}$

3. Western Blot Results

   Although our group's western blot result don't show obvious different, we found that active-caspase is found in western blot result from other group result, which suggested apoptosis induce pro-caspase to caspase.(Figure 3)

4 Discussions

Why our western blot result is not obvious? Many kind of reasons may result in this. Because of block, incubate together with other group, we think maybe the contest of protein few lower. Poor sample preparation and mistake on SDS-PAGE could decrease the concentration of tubulin and caspase in final loaded sample.

We only do the separate protein in based on the molecular weight in this experiment, but 2D SDS-PAGE involves the migration of polypeptides in 2 dimensions. More than based on molecular weight, polypeptides are separated according to isoelectric point too. The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine, arginine) and negatively (e.g. glutamate, aspartate) charged amino acids, with negatively charged amino acids contributing to a low isoelectric point and positively charged amino acids contributing to a high isoelectric point. 2D SDS-PAGE allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different isoforms of a particular protein. For example, a protein that has been phosphorylated.

5 Contributions

Wenhao Zhang and I finish these work together. Thanks to Dr. Zhao and TA. Jia's hard work to design experiments, prepare materials, give the lecture.

6 Figures

S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	Ctrl	Exp
0	0.077	0.205	0.275	0.383	0.475	0.554	0.689	0.446	0.417

Table 2. The absorbation at 562nm of samples.

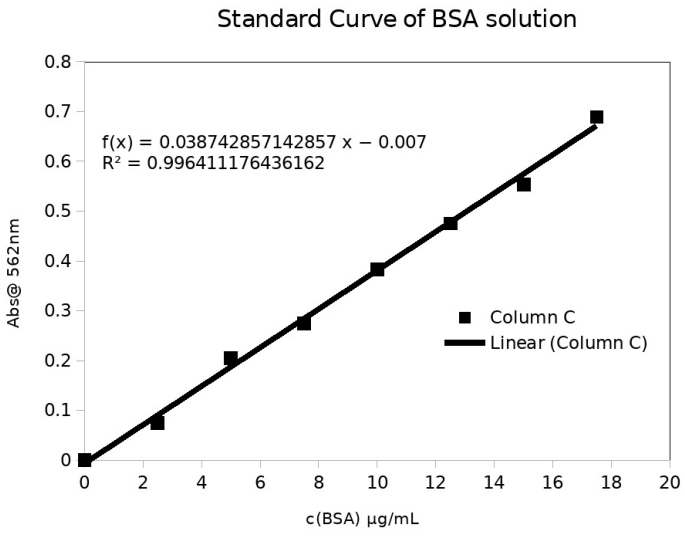

Figure 2. The standard curve of BSA solution.

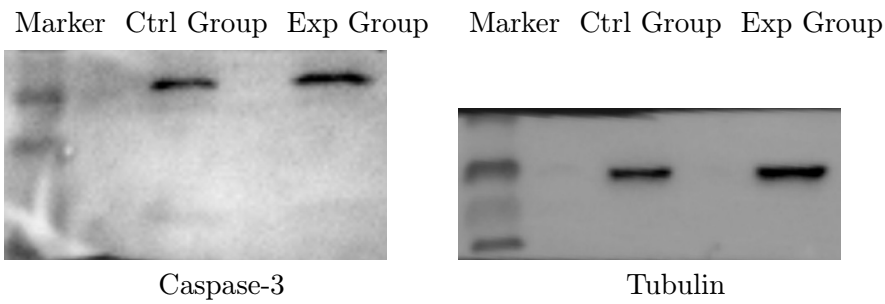

Figure 3. The western bloting result of caspase-3 and tubulin

7 Reference

- https://en.wikipedia.org/wiki/Western_blot
- Cell Biology Laboratory Manual Version V 2018
- Molecular Biology of The Cell, 6^{th}.