Cytoprotein Extraction, Quantification & Western Blotting

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1 Introduction

The growth, developmen, and maintenance fo multicelluar organisms depend only on the production of cells but also on mechanisms to destory 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 avtive site and cleave their target proteins at specific aspartic acids, which are therefore called **caspases**(c for cysteine adn asp for aspartic acid).

We plan to detect activated caspases-3 in apopotosis by western blot in this experiment. We design this experiment to find out the cytoprotein **caspases** and **tubulin** betweete apoptosis cell and normal cell by western blotting. For western blotting, we procudure 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 sperates and charasteric 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 phosphatase 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 duing 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.

BCA Protein Assay

Electrophoresis

Transfer Protein and Western Blotting.: The primary antibody and second antibody.

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 absorbation at 562nm in spectrophometer after using S_0 to blank. Form BSA absorbation data, we got the standard curve and concentration $c(\text{ctrl}), c(\text{exp})$ of cytoprotein of control group and experiment group.

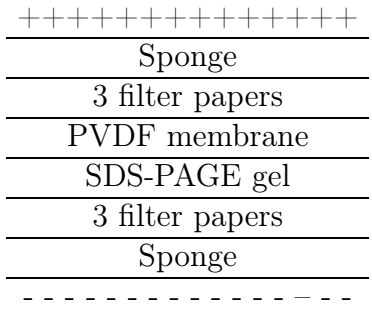
2.3 Western blotting

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 runging buffer(Tris 0.025M, Glycine 0.192M, SDS 0.1%, pH8.3), QuickBlock Western Blocking(Beyotime, P0252), Methanol, PageRuler^{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^{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 loadding 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^{TM} Prestained Protein ladder, two 10 μL samples on a precast gel. We ran SDS-PAGE at 30V for 10min, then rist to 200V for 30min. SDS-PAGE gel is took out and made a sandwich as follows:



PVDF membrane was immersed to methanol for 1 min to activited membrane before making sandwish. Setting up all parts and running 200mA for 2hr. Membrane is taken out, and was cut corner to indicate protein side. Memabrane was wased onece 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 substrade was added and taking image of membrane.

3 Results

1. The standard curve is

$$y = 0.03874x - 0.007$$

where y is the absorbation at 562nm, and x is the concentration of BSA solution.

2. The concentration of extration 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

   CASP succeful induce apostsis of cell.

4 Discussions

Why the CASP induce the apotsis?

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.

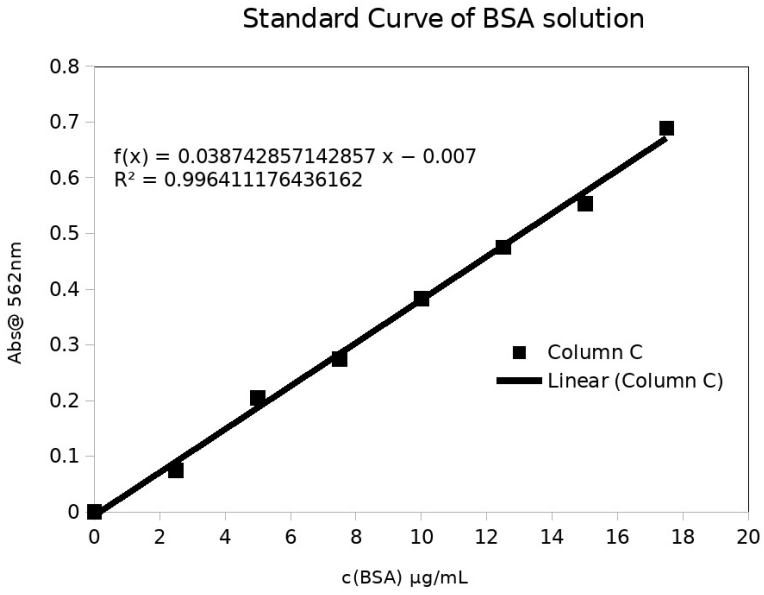
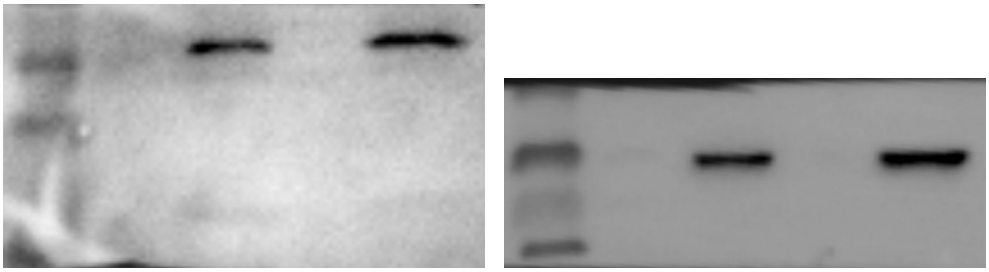


Figure 1. The standard curve of BSA solution.



CASP Tubulin

Figure 2. The western bloting result of CASP and Tubulin

7 Reference

- https://en.wikipedia.org/wiki/Western_blot
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