ภาคผนวก

BioRad Protein Assay

1. Preparation of protein standard (BSA)

1.0 mg/ml of BSA in dH_2O was prepared as a stock protein solution. Following 100 µg/ml protein dilutions were prepared from the stock protein solution.

1.1	0.0 mg BSA/ml	(tube no.1)
1.2	100 µg BSA/ml	(tube no.2)
1.3	250 µg BSA/ml	(tube no.3)
1.4	500 µg BSA/ml	(tube no.4)
1.5	750 µg BSA/ml	(tube no.5)
1.6	1,000 µg BSA/ml	(tube no.6)

2. Preparation of dilute dye reagent

Ten ml of dye reagent was pipetted to about 30 ml dH_2O in a measuring cylinder and adjusted to 50 ml with dH_2O. The diluted dye reagent was filtered through whatman filter paper No. 2.

3. Preparation of the sample supernatant

Following 100 µl sample dilutions were prepared from the sample supernatant :

(tube no. A)	3.1	100 µl sample supernatant
(tube no. B)	3.2	50 µl sample supernatant + dH_2O 50 µl
(tube no. C)	3.2	25 µl sample supernatant + dH_2O 75 µl

4. Pipetted 5 ml diluted dye reagent to each tube, gently mixed by vortex, and left it for at least 5 minutes.

5. Observed the blue color appeared, then in the order of color intensity, and measure the absorption at wavelenght, λ, 595 nm.

6. The measured absorptions were plotted against the protein concentration.

7. The protein concentration in sample supernatant could be estimated.

Remark dH_2O = deionized water

BioRad Protein Kit

Protein concentration was measuresd by the method described by Bradford (88) (Bio-Rad Protein Kit), using bovine serum albumin as a standard (0.2-0.9 mg). The assay mixture contained 20 µl of protein solution and 1.0 ml of diluted dye reagent (prepared by dissolving dye reagent concentrate and water in a ratio of 1:4 (v/v), the reagent was filtered through a Whatman No. 1 paper. After mixing and incubating for 5 min at room temperature, the absorbance was measured at 595 nm using a Shimadzu UV 160 Shimadzu spectrophotometer.

In the case of chromatographic separation, protein concentration was monitored by the measurement of absorbancy at 280 nm.

SDS-PAGE (I)

1. Reagents and gels for SDS-PAGE (Laemmli buffer system)

1.1 Stock solutions

A. Acrylamide/bis (30% T, 2.67% C)

14.6 g acrylamide

0.4 g N′N-bis-methylene- acrylamide

make to 50 ml with distilled water, filter and store at 4°C in the dark.

(30 days maximum)

B. 1.5 M Tris-HCl, pH 8.8

18.15 g Tris base

50 ml distilled water

adjust to pH 8.8 with 1 N HCl. Make to 100 ml with distilled water and store at 4°C.

C. 0.5 M Tris-HCl, pH 6.8

30 g Tris base

30 ml distilled water

adjust to pH 6.8 with 5-10 N HCl. Make to 50 ml with distilled water and store at 4°C.

D. 10% SDS

dissolve 10 g SDS in water with gently stirring, and bring to 100 ml with distilled water.

1.2 Separation gel preparation (bottom of lower gel)

-12% gel, 0.375 M Tris, pH 8.8

Distilled water	3.35	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
10% (w/v) SDS stock	0.1	ml
Acrylamide/bis (30% stock)	4.0	ml

Mix, then degas for 15 minutes at room temperature. Add fresh 50 µl of 10% ammonium persulfate, and 5 µl TEMED. Swirl gently and use immediately. 10 ml of the lower gel was obtained and only 4 ml gel was used for layering one glass plate. The setting time is at least 30-45 minutes.

1.3 Stacking gel preparation (upper gel)

-4.0% gel, 0.125 M Tris, pH 6.8

Distilled water	3.05	ml
0.5 M Tris HCl, pH 6.8	1.25	ml
10% (w/v) SDS stock	50	µl
Acylemide/bis (30% stock)	0.67	ml

Mix the mixture, then degas for 15 minutes at room temperature.

Add fresh 10% ammonium persulfate 25 µl, and 5 µl TEMES. Swirl gently and use immediately.

1.4 Sample buffer (store at room temperature)

0.5 M Tris-HCl, pH 6.6	2.0	ml
Glycerol	1.6	ml
20% (w/v) SDS	1.6	ml
2-Mercaptoethanol	0.8	ml
0.05% (w/v) bromophenol blue	0.8	ml
Distilled water q.s. to	8.0	ml

1.5 Running (electrode) buffer, pH 8.3

Tris base	1.5	g
Glycine	7.2	g
SDS	0.5	g
dH_2O q.s. to	100	ml

Store at 4°C, warm to 37°C before use if precipitation occurs.

Dilute 60 ml running buffer with 240 ml dH_2O for one electrophoresis run.

1.6 Fixing solution

Methanol	100	ml
Acetic acid	20	ml
Deionized water	80	ml

After electrophoresis, the gel was soaked with 100 ml of fixing solution in a tray and shake well for 10 minutes. Repeat this step two times.

1.7 Staining solution, freshly prepared

Quick-CBB	Staining solution A	30	ml
	Staining solution B	30	ml

The gel was soaked with 60 ml of staining solution in a tray and shake well for 30 minutes.

1.8 Destaining solution

Deionized water	75	ml
Methanol	15	ml
Acetic acid	10	ml

The gel was shaked in a cleaned tray containing 100 ml of destaining solution and shake well for 20 minutes.

2. Loading the chamber

All components of the instrument should be cleaned with a siutable laboratory detergent and rinsed throughly with distilled water before use.

- The short glass plate was put onto the long one, and the spacer was inserted between the two plates. The plates was then clamped using the casting chamber to fix the plate properly.
- Place the comb between the plates and mark at the point about one cm below the comb. This point will indicate the level of the lower gel. Take the comb out.
- Pipette the lower gel (ca. 4 ml) at either upper corner of the small plate. Add 1 ml dH_2O on the top.

- Leave the lower gel to setting for about at least 30 minutes. Then use syring to remove the water.

- Overlay the upper gel (ca. 1 ml) on the top of the lower gel. Insert the comb, and leave it to setting for about at least 1 hour.

- Take the comb out, and prepare the chamber.

- Put the chamber in the electrophoresis tank.

Add the running buffer to the chamber and tank. Observe whether there is any leak of the buffer from the chamber. If there is some leak, the chamber must be rearranged.

3. Sample preparation

- Pipette 5 µl sample, and 5 µl sample buffer. Mix them and boil in the boiling water for 3 minutes. Cool it and centrifuge at 15 x 100 rpm, flash. Then the sample is applied into the wells (which are made by the comb) using 50 µl micro-syringe.

4. Put the electrode to the 200 V Battery.

5. Leave the electrophoresis to develop for about 55 minutes.

6. After the electrophoresis, take the chamber out of the tank and take the gel out of the glass plate. Use the spatula to remove the upper gel. Rinse the lower gel with dH_2O.

7. The gel was then soaked in 100 ml ffixing solution with shaking for 10 minutes. Repeat the step two times.

8. The gel was soked in 60 ml staining solution with shaking for 30 minutes.

9. The gel soaked in 100 ml destaining solution with shaking for 20 minutes.

10. The gel was soaked in dH_2O with shaking for 20 minutes.

11. The gel was enveloped by the transparent film and then ready for the photography.