PROTOCOL: Bradford protein assay , SDS-PAGE, Coomassie staining, Western blot

Bradford protein assay

Material and chemicals:

Samples with protein
Bradford reagent
BSA concentration standard curve
Microtiter plate
Spectrophotometer

Preparation of solutions:

Bradford reagent

Coomassie Brilliant Blue G-250	100 mg
95% ethanol	50 ml
85% phosphoric acid	100 ml
dH_2O	fill up to 1 l

BSA concentration series
Dilute BSA to following concentrations: 100 µl/ml, 200 µl/ml, 300 µl/ml, 400 µl/ml, 600 µl/ml, 1000 µl/ml

Workflow:

1. Prepare samples of BSA for the concentration standard curve. Add 200 µl of Bradford reagent (blank) to the first well of the microtiter plate, then to each following well add 10 µl from the prepared dilutions and add 190 µl of Bradford reagent.

2. Prepare samples for your measurement. Dilute your protein samples 1 : 10 with water and then add 10 µl of each sample to a well of the microtiter plate (1 sample per well). Add 190 µl of Bradford reagent to the samples.

3. Let samples incubate for 10 minutes in room temperature.

4. Measure OD_{595}.

5. Benchmark your samples with the concentration standard curve and determine approximately the concentration of your samples.

SDS-Polyacrylamide gel electrophoresis

Material and chemicals:

Samples with protein
40% Acrylamide solution
Electrophoresis buffer
Sample buffer
1 M Tris-Cl (pH 8.7)
1 M Tris-Cl (pH 6.8)
10% APS, Temed

Preparation of solutions:

10 x Electrophoresis buffer

dH_2O	1 l
Tris	7.55 g
Glycine	36 g
SDS	2.5 g

2 x Sample buffer

1 M Tris-Cl (pH 6.8)	1 ml (fin. conc.: 100 mM)
20% SDS	2 ml (fin. conc.: 4 %)
Bromophenol blue	20 µl (fin. conc.: 0.2 %)
50% glycerol	4 ml (fin. conc.: 20 %)
ß-mercaptoethanol (78,13 M)	140 µl (fin. conc.: 200 mM)
dH_2O	fill up to 10 ml

1 M Tris-Cl

1. Dissolve 121.14 g of Tris in 1 l of dH_2O.
2. Divide into two bottles.
3. Adjust pH of the first bottle to pH 8.7 and of the second bottle to pH 6.8

10% APS

1. Dissolve 0.5 g of ammonium persulfate in 5 ml of dH_2O.
2. Store at 4 °C for a few weeks.

Gels

Chemicals	Running gel (12%)	Stacking gel (4%)
40% PAA	1,8 ml	500 µl
dH_2O	1,55 ml	3 ml
1 M Tris-Cl	2,25 ml (pH 8.7)	500 µl (pH 6,8)
10% SDS	60 µl	40 µl
10% APS*	60 µl	40 µl
Temed*	6 µl	4 µl

Notes:

- Add APS and Temed last, right before the pouring. After the addition, gels start to polymerize.

Workflow:

1. Calculate the volume of each sample that corresponds to 20 µg of protein. Transfer the calculated volume of sample to a clean Eppendorf tube. Add the 2 x sample buffer. To calculate the volume of the buffer, divide the sample volume by two.

2. Assemble the SDS-PAGE gel preparation apparatus. Pour distilled water into the apparatus to test if it does not leak.

3. Place the comb and make a mark with a sharpie, approximately 1 cm under the end of comb. Remove the comb.

4. Mix all the ingredients for the running gel. After addition of APS and TEMED, immediately proceed to step 5.

5. Pour the running gel to the sharpie mark. Carefully cover the gel with isopropanol using a syringe to remove bubbles. Let the gel polymerize (approximately 1 hour).

6. Remove isopropanol using a filter paper and rinse with water. Remove water using a filter paper.
7. Mix all the ingredients for the stacking gel. After addition of APS and TEMED, immediately proceed to step 8.

8. Pour the stacking gel to the rest of the volume. Add comb and let the gel polymerize (approximately 30 minutes).

9. Transfer the finished gel to SDS-PAGE performing apparatus. Fill the inner chamber with 1 x Electrophoresis buffer and check if it does not leak. The outer chamber does not have to be full. Remove the comb.

10. Temper the samples at 95 °C for 10 minutes. Centrifuge on 5000 g for 1 minute. Pipette the supernatant into the wells.

11. Connect to the electricity at 100 V. Electrophoresis takes about 3 to 4 hours. Follow the visible color marks - stop the electrophoresis when it reaches the bottom of the gel.

12. Proceed to Coomasie Briliant Blue staining or Wester blot.

COOMASSIE BRILLIANT BLUE STAINING

Material and chemicals:

SDS-PAGE gel with samples
Staining solution
Destaining solution

Preparation of solutions:

Chemicals:	**Staining solution**	**Destaining solution**
methanol	50 ml	25 ml methanol
dH_2O	40 ml	440 ml
glacial acetic acid	10 ml	35 ml
Brilliant Blue R-250	2.5 g	

Workflow:

1. Stain the gel for at least 2 hours (better: overnight)
2. Move stained gel to destaining solution and leave it there for 1 hour with shaking at room temperature.
3. After an hour change the destaining solution and again destain for 1 hour with shaking at room temperature.
4. Repeat this process one more time.
5. Now the gel is ready, but for the better results, you can change the solution again and leave it overnight.

WESTERN BLOT

Material and chemicals:

Gel with samples
PVDF membrane
Transfer solution
Ponceau solution
TBS
Blocking solution
TBS-T
Solutions with antibodies
SuperSignal™ West Femto Maximum Sensitivity Substrate

Preparation of solutions:

Transfer solution

Glycine	14.6 g (fin. conc. 190 mM)
Tris	3 g (fin. conc. 25 mM)
Methanol	200 ml (fin. conc. 20 %)
dH_2O	fill up to 1 l

Ponceau solution
For 100 ml dissolve 0.2 g of Ponceau S in 5 ml of acetic acid and add dH_2O

10 x TBS

NaCl	80 g
KCl	2 g
Tris	30 g
dH_2O	fill up to 1 l

adjust pH 7.4 with HCl, autoclave

TBS-T
1 x TBS with 0.5% of Tween-20

Blocking solution
1 x TBS-T with 5% of low-fat milk

Workflow:

1. PVDF membranes are rinsed in pure methanol for 10 seconds. The membrane should uniformly change from opaque to semi-transparent.

2. Afterwards equilibrate the membrane in the transfer solution for at least 5 minutes. Soak also the gel in transfer solution for 15 minutes.

3. Build up the transfer stack. Work in a tray with the transfer solution in it. Firstly open the blotting cassette. Place it accordingly to the direction of the transfer. Then place a sponge. Next place a piece of filter paper on the top of the sponge to cover it. Afterwards carefully place the gel on the filter paper. Cover the gel with pre wetted

PVDF membrane. Add filter paper and sponge again. With a small roller try to get out all of the air bubbles. Finally, close the cassette
(Notice: sponges, filter papers, gel, membrane and the whole sandwich have to be wet all the time!).

4. Insert the transfer stack into the blotting apparatus. Place it accordingly to the direction of the transfer. Add a cooling unit and stir into the chamber and fill it with cooled transfer solution.

5. Take a tray with ice and place it on a magnetic stirrer. Put the whole blotting apparatus inside the tray and turn on the stirrer.

6. Connect to the electricity at 100 V. Transfer takes about 1.5 hour.

7. Disassemble the apparatus and the stack (in a tray with transfer solution) to get the membrane. Wash the membrane in TBS-T

8. Check the transfer quality by staining the membrane with 1x Ponceau solution for approx. 5 minutes. Destain with water. Wash the membrane in TBS-T for 10 minutes.

9. Shake the membrane for 1 hour at room temperature in blocking solution (could be also done overnight).

10. Remove the blocking solution and add the first antibody which is prepared in 5% milk in TBS-T. Shake at room temperature approx. 15 hour (or overnight at 4 °C).
11. Wash the membrane in TBS-T 3 times for 10 minutes to remove the unbound antibodies.

12. Remove the TBS-T and add the second antibody which is prepared in 5 % milk in TBS-T. Shake 1 hour at room temperature.

13. Wash the membrane in TBS-T 3 times for 10 minutes to remove the unbound antibodies.

14. Visualize signals with SuperSignal™ West Femto Maximum Sensitivity Substrate.

15. Detect it with Kodak Image Station 2000 mM.