Western Blot Analysis of Chloroplast HSP70B in *Chlorella* Species

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[Abstract] Western blotting allows for the specific detection of proteins by an antibody of interest. This protocol utilizes isolation of total proteins protocol for *Chlorella vulgaris* prior to gel electrophoresis. After electrophoresis, the selected antibodies are used to detect and quantify levels of chloroplast HSP70B.

Materials and Reagents

1. Species

   Three *Chlorella* species were used: *C. vulgaris*, isolated from soil samples of Livingston Island, the South Shetland Archipelago, Antarctic; *C. vulgaris* strain 8/1, isolated in 1968 from thermal springs in the region of Rupite, Bulgaria, and cultivated in our laboratory since 1975 and *Chlorella kesslery* a mesophile, from the Trebon collection.

   Cultivation

   Chlorella species were cultivated on TAP (Tris Acetate Phosphate) medium under continuous light of 60 μmol/m^2/s and a temperature of 23 °C ± 0.1 °C in a Phytotron GC 400 growth chamber. The species were cultivated at this temperature because it is well known, that eurythermal algae, could be grown at a wide range of temperatures.
2. Rabbit polyclonal antibody HSP70B cytoplasmic (Agrisera, catalog number: AS06 175)
3. Goat anti-rabbit IgG(H&I) HRP conjugated (Agrisera, catalog number: AS09 602)
4. Coomassie brilliant blue G 250
5. Orthophosphoric acid (Valerus, catalog number: N 4420)
6. Trichloroacetic acid (TCA)
7. Bovine serum albumin (BSA) (Applichem GmbH, catalog number: 1391 0025)
8. Albumin fraction V (pH 7.0)
9. Medium Pure Nitrocellulose (NCM) (0.45 μm) (Bio-Rad Laboratories, catalog number: 162-0115)
10. Filter paper
11. Sponge

12. 4CN (4-chloro-naphthol) (Bio-Rad Laboratories, catalog number: N170-6535)
13. N,N´ N´ Tetramethylethylendiamine (TEMED) (Alfa Aesar, catalog number: N12536)
14. Laemmli sample buffer (see Recipes)
15. Reagent of Bradford (see Recipes)
16. 5x Laemmli buffer (see Recipes)
17. Running buffer (see Recipes)
18. Transfer buffer (see Recipes)
19. SDS-PAGE gel (see Recipes)
20. 30% Acrylamide/*N,N'*-methylenebisacrylamide (AA/MBA) (see Recipes)
21. 10% SDS (see Recipes)
22. 10% Ammonium Persulfate (see Recipes)
23. 1.5 M Tris HCl buffer (pH 8.8) (see Recipes)
24. 1.0 M Tris HCl buffer (pH 6.8) (see Recipes)
25. 4 M NaCl (see Recipes)
26. 1.0 M Tris HCl buffer (pH 7.5) (see Recipes)
27. 20% Tween 20 (see Recipes)
28. Blocking buffer (see Recipes)
29. Staining solution (see Recipes)
30. 5% CH_3COOH (see Recipes)
31. Washing solution (see Recipes)
32. 50 mM TBS-T buffer (see Recipes)
33. HRP color development solution (see Recipes)

Equipment

1. Motor
2. Silica quartz sand 0.6 mm (Valerus, catalog number: N 1760)
3. Centrifuge (Sigma-Aldrich, model: 1-15 K)
4. Electrophoresis chamber Transfer unit Hoefer miniVE electrophoresis and electrotransfer unit (Hoefer, model: SE300-10A-1.0)
5. Mini Rocker Shaker MR-1

Software

1. Image J program

Procedure

A. Cells lysis

1. Add 100 µl Lysys Solution (LS) to the pellet (Chankova *et al*., 2013b) transfer to a chilled mortar, add two spatulas of silica sand, grind in the mortar for 3 min, add 200 µl LS in the mortar to wash and transfer the material into an Eppendorf tube of 2 ml.
2. Centrifuge material from step 1 for 10 min at 14,500 *x g*.
3. Separate the supernatant and heat the supernatant for 5 min at t = 90 °C.
4. Centrifuge for 5 min at 14,500 *x g*.
5. Split the supernatant in 2 samples: The first one use for the determination of protein concentration; the second one keep at t = -20 °C.

B. Determination of protein concentration (Bradford)

1. Add 30 µl 20% TCA to 30 µl supernatant.
2. Centrifuge for 5 min at 14,500 *x g*.
3. Add 60 µl 0.1 N NaOH to the pellet and mix thoroughly. To obtain best result add twice 30 µl of 0.1 N NaOH.
4. Take 14 µl, add 86 µl 0.15 M NaCl and 3 ml reagent of Bradford.
5. Use calibration curve for quantity of protein (Table 1).
   For calibration curve:
   Stock solution – 0.5 mg/ml BSA
   Use Table 1 to determine every point of standard curve add 3 ml reagent of Bradford.

Table 1. Calibration curve for quantity of protein

N	BSA (μg)	BSA (0.5 mg/ml) vol (μl)	NaCl (0.15 M) vol (μl)
1.	0	0	100
2.	5	10	90
3.	10	20	80
4.	15	30	70
5.	20	40	60
6.	25	50	50
7.	30	60	40
8.	35	70	30
9.	40	80	20
10.	45	90	10
11.	50	100	0

C. Protein electrophoresis

1. Put about 100 ml of the 1x Laemmli buffer into cuvettes of electrophoresis module.
2. Remove the comb and rinse the wells with buffer of SDS-PAGE gel.
3. Pipet 10 μg protein into every well: adjust volumes so equal amount of protein is loaded (example: 10 μg protein are contained in 10 μl sample).
4. Put the rest buffer in a bath of electrophoresis chamber (the volume must be always above minimum).
5. Run electrophoresis using the following parameters: 120 V and 16 mA for 3.5 h.
6. When the electrophoresis is completed, remove the gel carefully.

   Note: The order of the dropping of the samples. Concentrated gel should be released.

D. Transfer of proteins on the NCM

1. Soak the gel for 15 min in buffer.
2. Soak sponge and filter paper for sandwich in transfer buffer.
3. Cut NCM. The size should be such as the size of the gel. Put NCM for 5 min in transfer buffer.

   Note: Mark the order of samples on the membrane! Label the membrane with a pencil.
4. Make a sandwich.
   a. The stack is assembled on the black cathode side (see Figure 1):
      i. Center a packing sponge on the black cathode side.
      ii. Center a packing sponge on the black catode (a).
      iii. Lay one piece of wet filter paper on the sponge (b).

iv. Position the equilibrate gels on the filter paper(c).
v. Lay the membrane on the gel (d).
vi. Lay one piece of wet filter paper on the membrane (e).
vii. Lay two packing sponges on the filter paper (f).
viii. A second transfer stack if added, is placed between these two sponges.

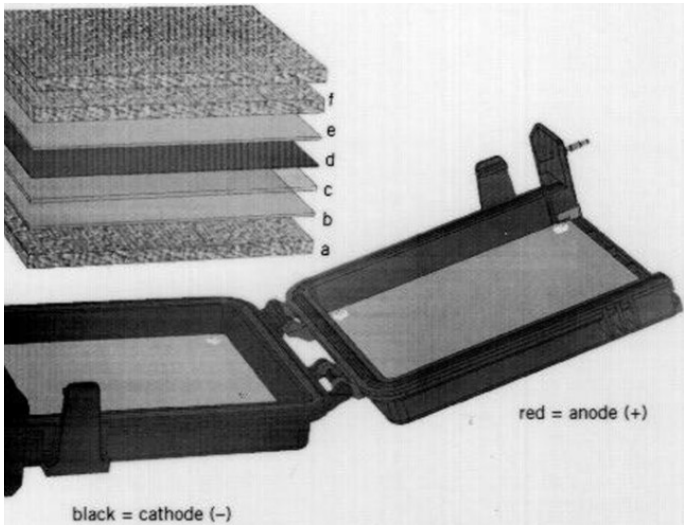

Figure 1. Assembling a transfer stack (*this is an original figure taken from the Technical Guide available at www. hoeferinc.com*)

b. Different parts of the sandwich press very well, to avoid bubbles.
c. Different parts of the sandwich should be well moistened. You can "roll" them with a tube.
   i. Close the apparatus. Put in a chamber transfer buffer.
   ii. Run blotting with the following parameters: 35 V and 250 mA for 2 h.

E. Western blot
1. After the transfer of proteins, place the membrane in blocking buffer at t = 4 °C. Incubate on a rocker platform for 1 h (following this step we have obtained the best results).
2. Place gels in staining solution for 4-5 h.
3. Wash for 3-4 h the gel with washing solution.
4. Dilute primary antibody in blocking buffer (1:10,000) and incubate according to manufacturer's instructions. Incubate on a rocker platform at t = 4 °C overnight.
5. Wash the membrane in TBS-T buffer on a rocker platform in a following way: twice for 2 min (2 x 2 min), after that twice for 10 min (2 x 10 min).

6. Prepare secondary antibody in blocking buffer (1:20,000) and incubate according to manufacturer's instructions. Incubate on a rocker platform at RT for 2 h.
7. Wash the membrane in TBS-T buffer on a rocker platform in a following way: Twice for 2 min (2 x 2 min), after that three times for 5 min (3 x 5 min).
8. Visualize using HRP Color Development Solution, 4CN according manufacturer's instructions.
9. Scan the membrane. Calculate protein amount using Image J program.

Recipes

1. Laemmli sample buffer
   2% SDS
   5% 2-mercaptoehtanol
   10% glycerol
   0.002%(w/v) bromophenol blue
   62.5 mM Tris HCl (pH 6.8)
2. Reagent of Bradford
   Dissolve 100 mg Coomassie brilliant blue G 250 and 50 ml 96% alcohol in a stirrer for 15 min. Add 94.5 ml 90% orthophosphoric acid.
   Add 900 ml deionized H_2O and stir gently.
   Filtering through a folded filter paper and make up to 1 L with deionized water.
   Keep in a fridge at t = 4 °C.
3. 5x Laemmli buffer
   15 g TRIS base
   72 g Glycine in 1 L deionized H_2O
4. Running Buffer
   Add 200 ml 5x Laemmli buffer + 10 ml 10% SDS to 1 L deionized H_2O
5. Transfer Buffer
   Add 200 ml 5x Laemmli buffer + 2 ml 10% SDS to + 200 ml ethanol to 1 L deionized H_2O
6. SDS-PAGE gel
   Note: Glass tiles should be cleared well with alcohol before preparing SDS-PAGE gel.
   a. Separating gel (12.5%) (Table 2)

Table 2. Preparing of separating gel solution

Number of mini-gels	1	2
Deionized H_2O	3.2 ml	6.4 ml
Acrilamide/bisacrilamide (30%)	4 ml	8.0 ml
1.5 M Tris HCl buffer, pH 8.8	2.6 ml	5.2 ml
10% SDS	100 µl	200 µl
10% APS	100 µl	200 µl
TEMED	10 µl	20 µl

i. Mix very carefully the components in a 50 ml Falcon tube to avoid bubbles.
ii. Insert separating gel between two glass plates of the chamber (about 1 cm below the boundary of tiles).
iii. Add deionized H_2O carefully as a thin film using a syringe and wait about 15 min.
iv. Carefully remove the water; Wipe the water drops in the ends with filter paper.

b. Stacking gel (Table 3)

Table 3. Preparing of 4% stacking gel solution

Number of mini-gels	1	2
Deionized H_2O	1,370 µl	2,740 µl
Acrilamide/bisacrilamide (30%)	330 µl	660 µl
1.0 M Tris HCl buffer, pH 6.8	250 µl	500 µl
10% SDS	20 µl	40 µl
10% APS	20 µl	40 µl
TEMED	2 µl	4 µl

c. Put the concentrated gel, insert the comb and wait until the gel polymerize.
d. For an electrophoresis is better to prepare about 1,250 ml 1x Laemmli buffer. It can be used twice.

7. 30% AA/MBA

   29.0 g + 1.0 g MBA dissolve in 72.5 ml deionized H_2O, make up the volume to 100 ml, filter using 0.45 µm filter

   Keep at t = 4 °C less than 1 month.

8. 10% SDS

   Dissolve 10 g SDS in 100 ml deionized H_2O

9. 10% Ammonium Persulfate

Dissolve 1 g in 10 ml deionized H_2O

Keep at t = 4 °C less than 1 month.

10. 1.5 M Tris HCl Buffer pH 8.8

    Dissolve 18.5 g Tris base in 80 ml deionized H_2O, adjust to pH = 8.8 with concentrated HCl and make up the volume to 100 ml.

11. 1.0 M Tris HCl Buffer pH 6.8

    Dissolve 12.114 g Tris base in 80 ml deionized H_2O, adjust to pH= 6.8 with concentrated HCl and make up the volume to 100 ml.

12. 50 mM TBS-T buffer

    1.0 M Tris HCl buffer (pH 7.5)

    200 mM NaCl

    0.1% Tween 20

13. 4 M NaCl

    Dissolve 23.376 g NaCl in100 ml deionized H_2O

14. 1.0 M Tris HCl buffer (pH 7.5)

    Dissolve 12.114 g TRIS base in 80 ml deionized H_2O, adjust to pH 7.5 with concentrated HCl and make up to the 100 ml.

15. 20% Tween 20

    20 ml Tween make up to 100 ml deionized H_2O.

16. Blocking buffer

    Dissolve 5% fatless dry milk in 100 ml TBS-T buffer.

17. Staining solution

    0.2% Coomassie Brilliant blue R- 250

    40% C_2H_5OH

18. 5% CH_3COOH

    Dissolve 2 g Coomassie Brilliant blue R- 250, 400 ml C_2H_5OH and 50 ml CH_3COOH and make up to 1 L with deionized H_2O.

19. Washing solution

    40% C_2H_5OH

    5% CH_3COOH

20. HRP Color Development Solution

    Dissolve 60 mg of 4-chloro-naphtol into 20 ml of methanol.

    Dissolve immediately before use and protect solution from light.

    Immediately prior to use, add 60 µl of ice cold 30% H_2O_2 to 100 ml TBS. Mix both solutions at RT. Use immediately.

References

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