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WesternBlot Paramecium bursaria (Pb)

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Protocol status: Working
We use this protocol and it's working

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Abstract

Western Blot protocol for Pb including all steps from the cell harvest to the final ECL stain. Culture density is an important factor that requires checking prior starting.

Protein extraction

1 To concentrate the culture, strain 100 ml of a dense Paramecium culture through a 40 micrometer strainer and split the volume over two 50 ml falcon tubes — 10m

2 Centrifuge the strained culture for 10 min at 800xg — 10m

3 Remove the supernatant from each falcon tube and wash the pellet with 5 ml of autoclaved NCL media.
NCL media: **https://doi.org/10.1073/pnas.210887411** — 3m

4 Centrifuge the washed culture for 10 min at 800xg — 10m

5 Remove the supernatant from each falcon tube but leave 1 ml to avoid disturbing the pellet. Transfer the 1 ml into 1.5ml Eppendorf tubes. — 3m

6 Centrifuge the sample for 10 min at 800 xg and remove the supernatant. Leave 50 ul to not disturb the pellet and pool the 2 tubes. You have now concentrated the sample 1:1000 (endvolume: 100 ul) — 10m

7 Add 200 ul of pre-warmed 2x SDS buffer (37C) supplemented with Proteinase Inhibitor (Roche; Cat. 4693116001), 2ul RNAseA (Qiagen; Cat. 19101) and 2ul DNAseI (Fisher Scientific; Cat. EN0521) to your concentrated sample — 3m

8 Incubate the sample at 37C for 30min — 30m

9 Optional: Make dilutions of your sample as needed using pre-warmed 6xSDS loading buffer (37C)

10 Optional: Centrifuge at max speed for 5 min and use supernatant to load the gel

Protein separation

11 Prepare 1x Running buffer (BioRad; Cat. #1610732)

12 Fill the gel chamber with '1x Running buffer'. Take a pre-made Mini PROTEAN TGX gel (BioRad Cat.# 4561096), rinse it briefly with water and load it into the holder. Carefully remove the comb. Remember to remove the green tape at the bottom.

13 Load the gel using 7ul for the protein ladder and 12 ul of protein extract. Fill empty wells with loading buffer

14 Run the gel for 60 min at 100V 1h

Transfer

15 Prepare 1x Transfer buffer, add 20% methanol (BioRad; Cat.# 1610734) and store at 4C.

16 Remove gel from holder and rinse under water. Use spatula to crack open each edge carefully. Optional: Cut a corner to mark the start of your gel

17 Soak the transfer membrane (Nitrocellulose Membrane, 0.2um BioRad Cat. #1620112), sponge, filter paper (Mini Trans-Blot Cat. #1703932) and gel in 1x transfer buffer for 15min on the shaker 30rpm at room temperature 15m

18 Assemble the 'transfer-cassette' and try to avoid any bubles. Use the small roller between each step carefully. Sequence: sponge/filter paper/gel/transfer membrane/filter paper/sponge. When closing the cassette try not to put to much pressure.

19 Put gel electrophoresis chamber into a tray on the magnetic stirrer. Fill the chamber with ice cold transfer buffer and add a spinbar to allow the buffer to move during the transfer. Fill the tray with ice to keep it cold during running and load the cassette into the chamber.

20 Run for 60 min at 100V 1h

Staining and blocking

21 Carefully remove the transfer membrane and rinse it in a square petri dish with ultrapure water.

22 Incubate the membrane with Pierce Reversible Protein Stain (ThermoScientific, Cat.# 24580) for 4 min 4m

23 Remove the stain and rinse the membrane 1x quick with Pierce Destain solution

24 Remove the Pierce Destain solution and rinse the membrane 2x with ultrapure water

25 Incubate the membrane for 5 min in Pierce Destain solution 5m

26 Remove the Pierce Destain solution and rinse the membrane 4x with ultrapure water.
Take a picture of the membrane.

27 Incubate the membrane for 5 min in ultrapure water 5m

28 Incubate the membrane for 2 min in Pierce Stain Eraser or until membrane is unstained 2m

29 Remove the Pierce Stain eraser and rinse the membrane 4x with ultrapure water

30 Incubate the membrane for 5 min in ultrapure water 5m

31 Incubate the membrane for 15 min in Denaturation Buffer (pre-warmed at 55C)
Denaturation buffer (50 mL): 10 mL 10% SDS, 350 uL beta-mercaptoethanol, 6.25 mL 0.5 M Tris HCl, 33 mL H2O (incubate buffer in 55C water bath beforehand). 15m

32 Incubate the membrane for 1 min in PBS-Tween (1x PBS + 0.1% Tween-20)
PBS: Sigma Aldrich P4417-50Tab
Tween-20:Sigma Aldrich P1379-250ml 1m

33 Incubate in 50 ml EveryBlot Blocking buffer (BioRad, Cat.# 12010020) overnight at 4C on rotor.

Antibody incubations

34 Wash membrane with PBS-Tween (1xPBS + 0.1% Tween-20)
PBS: Sigma Aldrich P4417-50Tab
Tween-20:Sigma Aldrich P1379-250ml 15m

35 Incubate membrane in 5 ml EveryBlot Blocking buffer (BioRad, Cat.# 12010020) with antibody#1 [1:250] for 3 hrs on the rotor 3h

36 Wash membrane with PBS-Tween (1xPBS + 0.1% Tween-20)
PBS: Sigma Aldrich P4417-50Tab
Tween-20:Sigma Aldrich P1379-250ml
15m

37 Incubate membrane in 5 ml EveryBlot Blocking buffer (BioRad, Cat.# 12010020) with antibody#2 [1:5000] for 1 hr in the dark on the rotor at room temperature
1h

Washing and staining
30m

38 Wash membrane in 5 ml PBS-Tween (1xPBS + 0.01% Tween-20), shake for 15 min at room temperature
15m

39 Wash in 2 ml PBS-Tween (1xPBS + 0.01% Tween-20) and shake for 5 min at room remperature
5m

40 Wash in 2 ml PBS and shake for 5 min at room temperature
5m

41 Prepare Pierce ECL dye mix (2.5 ml of each solution) and stain membrane for 5 min on shaker.
Pierce ECL Western (ThermoScientific Cat.#: 32106)
5m

42 Image membrane