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Cell Lysate Preparation & Immunoblotting Protocols

aasirvatham[1]

[1]Department of Biology, Misericordia University

aasirvatham

ABSTRACT

The purpose of this experiment is to investigate the effects of forskolin-mediated cAMP activation on the viability of LPS-treated Schwann cells. The immortalized rat RT4-D6P2T cell line (ATCC #CRL-2768) was cultured and received one of the following treatments: 0.1, 1, or 10 µg/mL of LPS, in N2 media (control) or N2 media supplemented with 2 µM of forskolin, for 1, 3, 12, or 24 hours. Cell lysates were prepared, and immunoblotting was performed to quantify changes in NF-κB, TNF-α, AKAP95, and cyclin D3 expression in response to the different treatment combinations.

To prepare RT4-D6P2T cell lysates (for three 6-well plates):

1 Aseptically culture immortalized rat RT4-D6P2T Schwann cells (ATCC, Cat #CRL-2768, Manassas, VA) in Dulbecco's Modified Eagle Medium (DMEM) (ATCC, Cat #30-2002, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher, Cat #16000044, Waltham, VA) and 1% penicillin/streptomycin (Pen-strep) (GIBCO, Cat #15140-015, Gaithersburg, MD)/amphotericin B (R&D Systems, Cat #B23192, Minneapolis, MN) at 37°C and 5% CO_2 in poly-L-lysine-coated dishes.

2 At 80% confluency, split and seed cells into DMEM (2 mL DMEM/well) in three 6-well plates at a density of ~300,000 cells/well.

3 Incubate cells in DMEM for 24 hours.

4 After 24 hours, aspirate the DMEM and wash each well 2-3x with 2 mL HBSS. After the last wash, add 2 mL N_2 media (DMEM/F12, no phenol red [Thermo Fisher, Cat #21041025, Waltham, MA] supplemented with 5 µg/mL insulin [Sigma, Cat #91077C, St. Louis, MO] and 100 µg/mL apo-transferrin [Sigma, Cat #T1147, St. Louis, MO]) to each well.

5 Incubate cells in N_2 media for 24 hours.

6 After 24 hours, prepare the forskolin-supplemented media by adding 10 µL of a 2 mM forskolin stock to 20 mL of N_2 media.

7 Add 2 mL of the appropriate medium to each well following the plate layout.

8 After adding the media, add the appropriate LPS dose to each well following the plate layout. For a 0.1 µg/mL dose of LPS, add 2 µL of a 100 µg/mL LPS stock OR 20 µL of a 10 µg/mL LPS stock. For a 1 µg/mL dose of LPS, add 2 µL of a 1 mg/mL LPS stock OR 20 µL of a 100 µg/mL LPS stock. For a 10 µg/mL dose of LPS, add 20 µL of a 1 mg/mL LPS stock.

9 Allow cells to incubate in the different treatment combinations for the required incubation time (3 hours).

10 After the required incubation time, remove the 6-well plates from the incubator and immediately put plates on ice. Keep plates on ice for the following steps.

11 Make ~3 mL lysis buffer by adding 2.970 mL IP lysis buffer (Thermo Fisher, Cat #87787, Waltham, MA) to 30 µL 10% 100X protease inhibitor cocktail (PIC) (Sigma, Cat #P8849, St. Louis, MO).

12 Wash each well 2x with non-sterile PBS.

13 After the last wash, add 100 µL prepared lysis buffer to each well.

14 Thoroughly scrape the surface of each well and pipette the lysates into the appropriately labeled Eppendorf tubes (on ice).

15 Agitate tubes at ~4°C for 30 minutes.

16 After 30 minutes, centrifuge the tubes at ~4°C and 10,000 RPM for 20-30 minutes (or until a pellet is visible).

17 After a pellet has formed in each tube, collect the supernatant.

18 Store lysates at -80°C for future use.

To run the gel (makes 2 small gels):

19 Dissolve ammonium persulfate (APS) (Sigma, Cat #A3678, St. Louis, MO) in DI H_2O to prepare a 0.1 mg/mL solution.

20 Clean small glass plates (Bio-Rad), glass molds, rubber sponges, and combs with 70% EtOH and assemble gel plates.

21 Insert 10-well comb (Bio-Rad) into apparatus and make small mark with marker on the glass just below comb, to indicate the bottom of the wells.

22 Make resolving gel consisting of 4.8 mL DI H_2O, 2.5 mL resolving buffer (VWR, Cat #M197, Radnor, PA) premixed with TEMED at 10 µL/mL, 2.6 mL Bisacryl (37.5:1) (VWR, Cat #M157, Radnor, PA), and 75 µL APS.

23 Fill the plates with resolving gel immediately up to the mark made on the glass from step 3. Add small amount of 0.01% SDS solution or 10% isopropyl EtOH to the top of resolving gel with a disposable pipette to prevent the gel from drying out.

24 Allow gel to polymerize for 15-25 minutes.

25 Once gel has fully polymerized, blot out the SDS/isopropyl EtOH with pieces of cardstock paper.

26 Prepare stacking gel by adding 4.5 mL stacking buffer, 0.5 mL Bisacryl, and 5 µL TEMED. (To make stacking buffer, use 137.5 mL of a 4X stock [VWR, Cat #M198, Radnor, PA], 550 µL TEMED, and 362 mL DI H_2O, and refrigerate).

27 Add the prepared stacking gel on top of the resolving gel layer and place comb in immediately. Wipe off any gel spillage on the outside of the glass, if necessary.

28 Allow gel to polymerize for 15-25 minutes.

29 Once gel has fully polymerized, disassemble gel apparatus and slowly remove comb from glass plates under running water.

30 Place gel assemblies into small box with the short glass plate facing inward and clamp into position.

31 Add 1X running buffer to the box, taking care to ensure that the electrodes are fully submerged in buffer. Make sure that the inner chamber is filled to the brim with running buffer, and fill 1/3 of the gel box with running buffer.

32 Add 4 µL marker (Bio-Rad Laboratories, Cat #161-0375, Hercules, CA) to designated gel walls and add 20 µL (maximum 25 µL) sample (RT4-D6P2T cell lysate) to designated gel wells with gel loading pipettes.

33 Use Bio-Rad Powerpac supply to run the gel.

34 Place lid on gel box and connect the leads to the power supply, taking care to see that the red electrode connects to the red terminal, and similarly with the black electrode.

35 Turn on electricity to 95V for the first 15-20 minutes, and once marker is about halfway down the gel, turn electricity to 105V for 45 minutes. Total gel run may take up to 2 hours.

36 Continuously check buffer levels. If it runs low, replenish the top of the chamber with buffer using a 10 mL syringe.

To prepare for transfer:

37 Cut 4 pieces of cardboard the same size as the small glass plate (10 cm x 8.5 cm).

38 Cut 2 PVDF membranes (Immobilon P – 0.45 µm, Cat #IPVH00010) to a dimension of 8.2 cm x 6 cm. Handle only the corners of the membrane with fingertips or forceps.

39 Make 500 mL 1X transfer buffer.

40 Soak all sponges in 1X transfer buffer.

41 Once the gel has completed its run (you will notice that the dye front is at the bottom), turn off the electrical unit.

42 Prepare the membrane by thoroughly soaking it in methanol followed by two rinses in DI H_2O. Then place the membrane in 1X transfer buffer for 5 minutes on a rocker.

43 Remove the gel from the cassette very slowly. With a flat spatula, remove the comb area of the

gel and any folded area on the bottom below the dye front if necessary.

44 In a Pyrex dish filled with 1X transfer buffer, assemble the sandwich as follows: black cassette down, sponge, cardboard, gel, membrane, cardboard, and sponge. Close and lock the sandwich.

45 Place the whole assembly in the chamber with a stir bar in the bottom. Make sure that the cassette in the transfer box has the black side facing black and the white side facing the red surface.

46 Place ice pack in back of transfer box.

47 Fill the chamber with 1X transfer buffer, enough to cover the sponges and place a stir bar at the bottom.

48 Place lid on transfer box, connect to the Bio-Rad power supply, and color match the electrodes with red on red and black on black. Transfer gel at 100V for exactly 1 hour.

49 After completion, wash the sponges and cassette along with the chamber. Discard the gel and the cardboard piece but save the membrane. Mark the marker lines on the membrane with a pen and allow membrane to dry. Store membrane at room temperature.

Western blotting:

50 Rehydrate membrane(s) in methanol, followed by two rinses in DI H_2O, if necessary.

51 Make 1000 mL Blotto by combining 100 mL 10X Tris/NaCl, 800 mL DI H_2O, 10 mL Tween 20, and 50 g Blotto Instant Milk.

52 Cut membrane into as many pieces as needed and soak membrane strips in Blotto on rocker for 1-1.5 hours.

53 Mix primary antibody following manufacturer's instructions.

54 After 1-1.5 hours of incubation in Blotto, remove Blotto from dishes, add primary antibody, and incubate on rocker for 1 hour at room temperature or overnight at ~4°C.

55 After 1-hour primary antibody incubation, perform five 5-minute washes (or three 10-minute washes). If the primary antibody incubation was in Blotto, use Blotto. If the primary antibody incubation was in TTBS, use TTBS.

56 Mix secondary antibody following manufacturer's instructions.

57 After the 5^{th} wash, add secondary antibody to dishes and incubate for exactly 1 hour.

58 After 1-hour secondary antibody incubation, perform five 5-minute washes (or three 10-minute washes). If Blotto was used, perform two washes in Blotto, followed by three washes in TTBS (or one wash in Blotto, followed by two washes in TTBS). If TTBS was used, perform all washes using TTBS.

59 Mix equal amounts of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Cat #34580, Waltham, MA) and stable peroxide solution in 15 mL conical wrapped in aluminum foil. These chemicals are light-sensitive.

60 Cut sheet protector into the appropriate size for each membrane strip.

61 After the last wash, add luminol solution and incubate on a rocker for 1 minute in the dark.

62 Place each membrane strip into a sheet protector and place a small drop of fluorescent paint onto the ink lines (which designates the marker bands).

63 Analyze protein bands using Image Lab 6.1 software (Bio-Rad Laboratories, Hercules, CA). Follow instructions as specified for protein target.