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Western Blot Protocol - Updated for Lrrk2, Nitrotyrosine, etc.

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We use this protocol and it's working

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Abstract

This protocol outlines a reliable workflow for the detection of target proteins by Western blot, with specific adaptations for LRRK2 and nitrotyrosine. It is suitable for a variety of proteins and sample types.

Prepping Samples

1 Turn on heat block (95-100 degrees Celsius).

2 Thaw samples, protein ladder, 4X Nupage loading buffer (reducing – 5% Beta-mercaptoethanol).

2.1 Note – protein samples for Nitrotyrosine are non-reducing and do not contain BME.

2.2 Nitrotyrosine samples are 75ug of protein, all other WB samples are typically 40ug.

Running Gel

3 Heat samples for 8 min.

4 Put on ice for 5 min.

5 Spin down samples briefly.

6 Place gel (4-12%) in gel box (adding 1 plastic filler to make the hold secure or another gel). Shorter side faces towards you.

7 Fill running cassette with 1X MOPS Running Buffer for LRRK2 or 1X MES Running Buffer for other proteins and let it flow throughout the gel box.

8 Load samples (15 uL max in 15 well gels, 30 uL for 12 well and 50 uL for 10 well), add 5uL of loading buffer to empty wells.

9 Make sure the electrodes are properly secured with lid and turn on powerpac.

10 Set voltage to 168 V and run for 35-50 min (can take longer depending on sample and required protein). Watch to make sure bubbles appear where the electrode is located

(base of unit).

11 Before leaving soak several sponges in transfer buffer. Use a glass slide holder to weigh down the sponges in the liquid. Also, make sure you have enough filter paper and PVDF membranes.

12 When run is complete turn off powerpac and carefully disassemble the unit. Do not let the gel dry.

Transfer

13 Pour a small amount of methanol in plastic Tupperware.

14 Setup the transfer as follows:

14.1 Place transfer cassette inside large Tupperware and pour small amount of FRESH transfer buffer.

14.2 Put one sponge in cassette and smooth out.

14.3 Place 1 filter paper and smooth out with roller.

14.4 Open gel case carefully, remove excess gel on wells and place in cassette carefully. Gels can easily break, especially at the top of the gel.

14.5 Use tweezers to unwrap PVDF membrane and place in methanol for a few seconds with swirling.

14.6 Transfer membrane on top of gel making sure to not touch the membrane with gloves or hands.

14.7 Place another 1 filter paper over top of membrane carefully, using roller to smooth out bubbles.

14.8 Place more sponges until they just barely surpass the base of the cassette and then place the lid of cassette on top.

14.9 Squeeze to close while holding over the base of the running/transfer container. And tighten the unit.

14.10 Pour FRESH TRANSFER BUFFER to the top of the transfer cassette and ddH2O to fill the outside of chamber.

14.11 Close lid securely and turn on powerpac. Set voltage to 30 V for 70-90 min (70 min for most proteins, 90 min for large proteins like Lrrk2).

Blocking

15 Once run is complete, carefully disassemble the unit, and remove the sponges carefully to look at the membrane transfer. If it looks like the transfer is incomplete (ladder lanes still present in the gel) then reassemble, adding more transfer buffer and ddH2O and run at 30 V for an additional 30 min.

16 If complete, transfer the membrane, using tweezers, to a plastic container and rinse once with 1X TBST.

17 Add 5-10 mL/membrane of 5% BSA or milk (depending on antibody) and rock for 1 hr to block membrane.

Primary Antibody

18 After one hour, if possible/required based on molecular weights, cut the membrane in half with a blade. For example for Lrrk2 (252kDa) and actin (43kDA) cut the blot at the 50kDa ladder marking and put the two halves in two separate cassettes. This way you can image both halves of the blot on one day instead of reblotting over two days to obtain the actin housekeeping blot.

19 Add 5-10 mL of primary antibody diluted in 5% BSA or milk (depending on antibody) and seal with parafilm (or put in cassette with hinges) (and do the same with second blot, if applicable).

20 Bring to 4 degree room and rock overnight.

21 Next day, carefully pour primary antibody into a labeled 15 or 50 mL tube (this antibody can be reused).

Secondary Antibody

22 Wash 3× 5 min with 1X TBST.

23 Add 1:10,000 (or required dilution) secondary antibody in blocking solution (5% milk or BSA).

24 Rock for 1 hr at room temperature.

25 Remove secondary (do not keep this antibody).

26 Wash 2X 15 min with TBST.

Developing with Machine: Chemidoc (LRRK2 and Most Proteins)

27 Bring: WestFemto reagents, tweezers, clear sheets, kim wipes, blots, p1000, and tips to ChemiDoc.

28 Remove TBST from the blot.

29 Combine 500uL WestFemto reagent A and 500uL WestFemto reagent B and add to the blot.

30 Rock the blot and using the p1000 add the combined reagents all over the blot.

31 Using tweezers, add the blot between the clear folder.

32 Place in the ChemiDoc, and select Blots>Chemi.

33 Auto-expose.

34 If you would like, expose for 5 seconds and upwards (sometimes I have done 300 seconds when necessary) until imaged as desired.

35 Next, without moving blot at all, Select Blots>Colormetric to image the ladder.

36 Save the ChemiDoc images for densitometry analysis.

Developing with Film (Nitrotyrosine)

37 Mix equal volumes of ECL reagents in a 15 mL tube and use a 1 mL pipet to add 4 mL over the entire surface of the membrane.

38 Rock for 3 min.

39 Transfer to clear sheet protectors in the film-developing cassette and go to developer room. (bring films, cassette and timer, developer film CL-XPosure Film, Thermo cat# 34091).

40 Do not use any light in the room (no phones, use the timer you brought).

41 Take only one film out at a time and crease one corner to help orient film.

42 Expose for 2, 5, 10, 30 sec. and then run through machine (make sure to press the activating button to turn on the machine). Wait until buzzer sounds before adding additional films or opening the door.

43 If exposure is not long enough, increase to 1, 2, 5 and even 30 min. After 30-60 min the signal will be less strong.

44 After exposing, bring all materials down to lab and correctly label wells, exposure time, date and always include concentrations of primary and secondary antibodies for future reference.

45 Remove membrane, using tweezers, from sheet protectors and wash 1X 5 min with 1X TBST.

Reblotting Procedure

46 Pour off the ECL and wash with TBST for 5 minutes (store in fresh TBST after the wash if stopping at this step).

47 Add 5-10 mL of 1X ReBlot and mix for 15 min.

48 Wash 1X 5 min with 1X TBST and repeat steps 18-39.

TBST Recipe

49 50mL 1X TBS

50 450mL milliQ H2O

51 500uL of Tween-20