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Western Analysis used in Oxidative Stress Protocols V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.8bbhsin

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ABSTRACT

Summary:

This is the general protocol used for western analysis of samples from the Oxidative Stress Protocols. There are no specific antibodies described for use, rather a general procedure for creating the western. See the specific assay for the details about the antibodies used.

Diabetic Complications:

EXTERNAL LINK

https://www.diacomp.org/shared/document.aspx?id=31&docType=Protocol

MATERIALS

NAME	CATALOG #	VENDOR
12.5% Acrylamide gel stock solution		Sigma Aldrich
10% Ammonium persulfate (APS)	View	Sigma Aldrich
TEMED	View	Sigma Aldrich
2-propanol	View	Sigma Aldrich
10X TBS	View	Sigma Aldrich
10x Sample Buffer		
10X Running Buffer		
10X Transfer Buffer		
5% Fat Free Milk	Grocery Store	
Gel loading tips	View	Fisher Scientific
Nitrocellulose		Schleicher & Schuell

NAME	CATALOG #	VENDOR
ECL kit		Amersham

MATERIALS TEXT

Reagent Preparation:

10X TBS	RIPA BUFFER + INHIBITORS
1 Liter	**10mL**
12.1g Tris Base 87.7g NaCl 950mL ddH2O pH to 8 with conc. HCL Bring final volume up to 1000mL	10mL Ripa 10µL 0.1 trypsin units/µL aprotinin 10µL 10mg/ml leupeptin 20mL 50mg/ml PMSF (phenylmethylsulfonyl fluoride) 100µL Na deoxycholate
TBST (1L)	**Milk** (100mL)
100mL 10X TBS 900mL ddH2O 10mL 10% TWEEN-20	10mL 10X TBS 90mL ddH2O 1mL Thimerosol 1% 1mL TWEEN-20 10% 5g milk
500mL TBS	**10% TWEEN-20**
50mL 10X TBS 450mL ddH2O	10mL TWEEN-20 90mL ddH2O

Separating Gel Preparation:

1 gel: 10mL 12.5% gel stock
50µL 10% APS
7µL TEMED

Stacking Gel Preparation:

2 gels: 5mL stacking gel stock
30µL 10% APS
5µL TEMED

1 Wash & dry plates.

2 Assemble rig and fill plates with H_2O to check for leaks.

3 Pour off water and wipe dry with kimwipe.

4 Load gel to about the top of the door.

5 Add 2-propanol to cover the edge.

6 Wait ~ 40 minutes to polymerize.

7 Thaw samples on ice.

8 When gel is ready, pour off 2-propanol and rinse with H_2O.

9 Remove excess H_2O with a kimwipe.

10 Prepare and load stacking gel and insert comb making sure there are no bubbles under the teeth.

11 Put a beaker of water on the hot plate to boil.

12 Prepare samples:

♦ Plasma - dilute 2µL plasma in 198µL (1:50) RIPA buffer + inhibitors in a labeled screw top tube. Sonicate on 5. Pull off 10µL for Protein analysis. Add 38µL 10X samples buffer to the 190µL lysate.

♦ DRG and Sciatic nerve - **DRG** - After removing 4 DRG for TRAP assay, pool the remaining DRG in a labeled screw top tube. **Sciatic nerve** - Place 1 sciatic nerve into a labeled screw top tube.

Add 110µL RIPA buffer + inhibitors. Sonicate on 8 on ice. Freeze samples, thaw and run through a 1mL syringe with a 26g needle. Repeat Freeze, thaw and running through syringe. Pull off 10µL for Protein analysis. Add 20µL 10X sample buffer to the 100µL lysate.

13 Label screw top tubes for markers.

14 Do protein analysis on samples. Generally load 20 to 50µg

15 Add 2µL 10X sample buffer to 10µl rainbow protein marker. (times x for x # of gels)

16 Boil samples and markers for 5 minutes and cool.

17 When gel is done gently remove combs.

18 Assemble rig with short plate on the inside, press down and close doors.

19 Fill inside chamber with running buffer to about ½ way between top of sm & lg plate and make sure there are no leaks.

20 Pour more running buffer into outside of rig to the bottom of the gate.

21 Load rainbow protein marker and samples.

22 Set volts @ 200 and run for 50-60 minutes.

23 Remove gel from rig, remove wells and soak gel in transfer buffer for 15 minutes.

24 Cut and label nitrocellulose membrane to size and soak in transfer buffer.

25 In another dish, soak 2 fiber pads and 2 pieces of whatman paper for each gel.

26 Assemble the sandwich with black side down in transfer buffer, making sure there are no bubbles between each layer put 1 fiber pad, 1 whatman paper, gel, nitrocellulose, 1 whatman paper, and 1 fiber pad.

27 Put a stir bar in the bottom of the rig and place the sandwich in the transfer unit with the black part in the back. (Protein runs from black to red, to the membrane)

28 Fill the ice pack and place behind the sandwich.

29 Fill the unit with 1X transfer buffer until the ice pack floats or the top of the lower ledge.

30 Transfer at 100V for 1 hour (100kd-30kd) or 69V for very low proteins

31 Rinse the membrane in 1X TBS for 10 minutes.

32 Block overnight @ 4º or at RT for 2 hours in TBST/milk for polyclonal antibodies or TBST/BSA for mAbs.

33 Quick rinse once with TBST.

34 Incubate 2 hours at RT or overnight @ 4º in primary antibody in TBST/milk or TBST/BSA on rocker. (Primary antibody can be re-used)

35 Wash 3x's for 5 minutes with TBST.

36 Incubate for 2 hour in secondary antibody in TBST/milk or TBST/BSA.

37 Quick rinse once with TBST.

38 Wash 3x's for 5 minutes each in TBST.

39 Wash 20 minutes in 1X TBS.

40 In a 15mL conical tube, develop with small cell signaling bottles using 9mL H_2O and 500µL of each reagent. Expose for 1 minute.

41 Develop film.