VERSION 2

OCT 16, 2019

OPEN ACCESS

DOI:

dx.doi.org/10.17504/protocols.io.8bbhsin

External link:

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

Protocol Citation: Eva Feldman 2019. Western Analysis used in Oxidative Stress Protocols. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.8bbhsin>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: Oct 16, 2019

Last Modified: Oct 16, 2019

PROTOCOL integer ID:
28739

Western Analysis used in Oxidative Stress Protocols V.2

Eva Feldman¹

¹University of Michigan - Ann Arbor

Diabetic Complications Consortium

Tech. support email: rmcindoe@augusta.edu

Lili Liang

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:

Neuropathy

MATERIALS

MATERIALS

⊗ 12.5% Acrylamide gel stock solution **Sigma Aldrich**

⊗ 10% Ammonium persulfate (APS) **Sigma Aldrich**

⊗ TEMED **Sigma Aldrich**

⊗ 2-propanol **Sigma Aldrich**

⊗ 10X TBS **Sigma Aldrich**

⊗ 10x Sample Buffer **Contributed by users**

Keywords: Biochemical Measures of Neuropathy, diabetic neuropathy, Western Analysis

✂ 10X Running Buffer **Contributed by users**

✂ 10X Transfer Buffer **Contributed by users**

✂ 5% Fat Free Milk **Contributed by users** **Catalog #Grocery Store**

✂ Gel loading tips **Fisher Scientific**

✂ Nitrocellulose **Schleicher & Schuell**

✂ ECL kit **Amersham**

Reagent Preparation:

10X TBS	RIPA BUFFER + INHIBITORS
1 Liter	10mL
12.1g Tris Base 87.7g NaCl 950mL ddH ₂ O 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 ddH ₂ O 10mL 10% TWEEN-20	10mL 10X TBS 90mL ddH ₂ O 1mL Thimerosal 1% 1mL TWEEN-20 10% 5g milk
500mL TBS	10% TWEEN-20
50mL 10X TBS 450mL ddH ₂ O	10mL TWEEN-20 90mL ddH ₂ O

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 H₂O 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 H₂O.
- 9 Remove excess H₂O 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 $\frac{1}{2}$ 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 @ 4° 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 @ 4° 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 H₂O and 500µL of each reagent. Expose for 1 minute.
- 41** Develop film.