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Neuropathy Phentoyping Protocols - Immunohistochemistry for Tissue Sections V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.7rfhm3n

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ABSTRACT

Summary:

Phenotyping of Rodents for the Presence of Diabetic Neuropathy
In man, the development of diabetic neuropathy is dependent on both the degree of glycemic control and the duration of diabetes. Diabetic neuropathy is a progressive disorder, with signs and symptoms that parallel the loss of nerve fibers over time. Consequently, assessments of neuropathy in mice are not performed at one time point, but are characterized at multiple time points during a 6 month period of diabetes. The degree of diabetes is evaluated in 2 ways: tail blood glucose measured following a 6 hour fast and glycated hemoglobin levels. The initial degree of neuropathy is screened using the methods discussed below. Detailed measures of neuropathy are employed when the initial screening instruments indicate a profound or unique phenotypic difference. This document contains protocols used by the DiaComp staff to examine and measure diabetic neuropathy at the whole animal, tissue and cellular levels.

Diabetic Complication:

EXTERNAL LINK

https://www.diacomp.org/shared/document.aspx?id=54&docType=Protocol

MATERIALS TEXT

Reagent List:

Buffers
- Phosphate buffer (PB) pH 7.2, 0.1 M
- Phosphate buffered Saline (PBS) pH 7.2, 0.1 M, 150 mM NaCl
- Tris, pH 7.2- 7.6, 0.1 M
- 0.1% TX100 in PB

Fixatives:
- 4% paraformaldehyde
- 2% paraformaldehyde
- 4% paraformaldehyde with 0.5% glutaraldehyde (for TEM)
- 1:1 acetone: methanol

Primary Antibodies: Working dilutions vary

Secondary Antibodies: 1:200 to 1:500

Biotinylated
Avidin HRP kit
Fluorochrome labeled

Counterstains:

Hematoxylin
Eosin
Methyl Green
Bis-benzimide (DAPI)

Other Chemicals:

3'3-diaminobenzidine
glucose oxidase
ammonium chloride
β-D Glucose
Ethanols 50, 70, 95, 100%
Hemo-D
Normal serum
Non-fat dry milk
Bleach
H_2O_2

Mounting Media:

Permount
Prolong
Gelmount

Glassware:

Square Wheaton dishes
Slide racks and handles
Coplin jars
Beakers
Microfuge tubes

1 **General Concepts:**

• Keep buffers consistent through fix, rinses, antibody dilutions and chromogen development.

• Antibodies are sticky, always use plastic tips and tubes for storing, diluting and pipeting antibodies.

• Check slides after each major step in the protocol to make sure sections are still stuck to the slide. There is no point in continuing if the sections have all been removed due to over zealous washes.

2 **Cryosections:**

1. Remove slides from freezer, air dry. (warm plate)

2. Ring sections with PAP pen, if tissue was not fixed prior to embedding and sectioning, fix sections for 10-30 min, 4ºC
   - ♦ 4% paraformaldehyde
   - ♦ 2% paraformaldehyde
   - ♦ 1:1 acetone: methanol, ice cold

3 **Paraffin Sections:**

1. Deparaffinize in Hemo-D.

2. Hydrate through ethanol 100, 100, 95, 95, 70, 50, distilled water.

3. Ring sections with PAP pen, carefully, do not let sections dry.

4 **Prepare Humid Chamber:**

1. Hydrate in buffer 5 min.

2. Rinse in 0.5% H_2O_2 in buffer (5 ml 30% in 300 ml buffer), 10 min (for immunofluorescence skip and go to blocking)

3. Rinse 3 X 10 min in buffer.

4. Permeabilize and block 10 minutes:
- ♦ 0.1% TX100 in buffer
- ♦ 2% non-fat dry milk
- ♦ 1% serum (serum from the animal in which the secondary antibody is raised e.g. 1º is rabbit, 2º**goat** anti-rabbit, block with normal goat)

5. Diluted primary antibody in buffer containing 0.1% TX100

6. Remove slides from blocking solution, do not rinse, dab away excess fluid with filter paper, place 1º (20-40 ul) in PAP pen ring. Dilutions are antibody specific, check antibody book for suggested dilutions.

7. Always wrap parafilm around the edge of the incubation chamber to seal:
- ♦ Incubation times: 1º 's overnight 22ºC, parafilm,
  36-48 hours 4ºC, parafilm
  2-4 hours 37ºC, lean toward 50-60 µl

8. Rinse in buffer 3 X 10 minutes.

5 **Immunofluorescence:**

1. Turn off overhead fluorescent lights to decrease quenching.

2. 2º antibody labeled with red or green fluorochrome.

3. Incubate 1 hour 22ºC.

4. Rinse 3 X 10 min buffer.

5. Counterstain with Bis-benzimide 1ug/ml buffer, 5 min.

6. Rinse in buffer.

7. Cover with Prolong (see Prolong SOP)

6 **Immunoperoxidase:**

1. 2º antibody labeled with biotin.

2. Incubate 1 hour 22ºC.

3. Rinse 3 X 10 min buffer.

4. Incubate in Avidin HRP I hour 22ºC **Prepare this solution 1 hour prior to use, it needs to equilibrate**

5. Rinse 3 X 10 min buffer.

6. DAB

7. Rinse 3 X 10 min in buffer, collect first rinse and add bleach to denature DAB (DO NOT PUT BLEACH ON THE SLIDES)

8. Dehydrate through graded ethanol 50, 70, 95, 95, 100, 100% and 2 changes of Hemo-D Counterstain.

9. Mount coverslips to slides with Permount.