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# Neuropathy Phentotyping Protocols - Immunohistochemistry for Tissue Sections V.2

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**Protocol status:** Working

We use this protocol and it's working

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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:**



Neuropathy



## Materials

### 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  
 $\beta$ -D Glucose  
Ethanol 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, 4°C
  - ♦ 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<sub>2</sub>O<sub>2</sub> 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. 1° is rabbit, 2° **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 1° (20-40  $\mu$ l) 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: 1° 's overnight 22°C, parafilm,  
36-48 hours 4°C, parafilm  
2-4 hours 37°C, lean toward 50-60  $\mu$ l

8. Rinse in buffer 3 X 10 minutes.

## 5 **Immunofluorescence:**

1. Turn off overhead fluorescent lights to decrease quenching.

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

3. Incubate 1 hour 22°C.

4. Rinse 3 X 10 min buffer.

5. Counterstain with Bis-benzimide 1 $\mu$ g/ml buffer, 5 min.

6. Rinse in buffer.

7. Cover with Prolong (see Prolong SOP)

## 6 **Immunoperoxidase:**

1. 2° antibody labeled with biotin.

2. Incubate 1 hour 22°C.

3. Rinse 3 X 10 min buffer.

4. Incubate in Avidin HRP 1 hour 22°C \*\*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.