Sep 27, 2019 Version 2

Biochemical Measures of Neuropathy - Hydrogen Peroxide Assay V.2

DOI
dx.doi.org/10.17504/protocols.io.7rghm3w

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.7rghm3w

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

Protocol Citation: Eva Feldman 2019. Biochemical Measures of Neuropathy - Hydrogen Peroxide Assay. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.7rghm3w**

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

Created: September 27, 2019

Last Modified: September 27, 2019

Protocol Integer ID: 28168

Keywords: Biochemical Measures of Neuropathy, diabetic neuropathy, Hydrogen Peroxide , biochemical measures of neuropathy, neuropathy, oxidative stress, increased levels of glucose overload mitochondria, hydrogen peroxide assay summary, antioxidant capacity, hyperglycemia, glucose overload mitochondria, axons of the peripheral nervous system, diabetic complication, biochemical measure, metabolic change, excess glucose, peripheral nervous system, neuron, flow of excess glucose, cellular pathway, reactive oxygen species

Abstract

Summary:

Oxidative stress is highly correlated with the metabolic changes caused by hyperglycemia. Increased levels of glucose overload mitochondria and result in the production of reactive oxygen species (ROS). In addition, the flow of excess glucose through cellular pathways decreases the cell’s normal ability to detoxify ROS. As a result, the neurons and axons of the peripheral nervous system contain increased levels of ROS and decreased antioxidant capacity. The following assays are used to measure these changes in rodent models of diabetic neuropathy.

Diabetic Complication:

Neuropathy

Materials

MATERIALS

Amplex Red Hydrogen Peroxide Assay Kit **Catalog #** A-12212

HPLC-grade water

Reagent Preparation:

Amplex Red reagent: Prepare a ~20 mM stock solution. Bring DMSO and 1 vial of Amplex Red reagent to room temp. Just prior to use dissolve the Amplex Red reagent in 200 µL DMSO. Store stock solution an –20ºC, protected from light.

Reaction Buffer (5X) (0.25M sodium phosphate, pH 7.4): Dilute 5 mL of Reaction buffer in 20 mL of deionized water.

HRP (Horseradish peroxidase): Dissolve 1 vial of HRP in 1 mL of 1X Reaction Buffer (200 U/mL). After use divide remaining stock into small aliquots and store frozen at –20ºC.

20mM H_2O_2: Dilute (check bottle for %) 17.9 µL H_2O_2 (3.8%)in 982.1 µL dH_2O. (Check label for exact concentration) (23 µL 3% H_2O_2 into 977 µL dH_2O) Use promptly.

Resorufin, sodium salt: add 1mL dH_2O to a vial of resorufin solid. (2 mM stock). Store at –20ºC, protected from light.

Troubleshooting

Sample Preparation:

1 Prepare stock solutions above for Amplex Red Reagent, Reaction Buffer, HRP, 20 mM H_2O_2 and Resorufin, sodium salt.

2 Prepare H_2O_2 standard curve. Label tubes 1-5.

Add 999 µL 1X Reaction Buffer to tube 2.
Add 100 µL 1X Reaction Buffer to tubes 2 – 5.
Add 1 µL of 20 mM H_2O_2 working dilution prepared above to tube # 1 to produce concentrations of 20 µM.
Take 100 µL from tube 1 and add to tube 2.
Take 100 µl from tube 2 and add to tube 3.
Take 100 µl from tube 3 and add to tube 4.
Tube 5 is dH_2O only.
(Final concentration will be twofold lower, 0 to 10 µM.)

3 Dilute samples in 1X Reaction Buffer.

4 Pipette 100 µL of diluted standards, controls (if any) and samples into wells. (For DRG we used 25 µL)

5 From 20 µM stock solution of Amplex Red reagent - Prepare 400 µM dilution containing 2 U/mL HRP by adding 20 0µl of Amplex Red stock solution and 100 µL of 200 U/mL HRP stock solution to 9.7 mL of 1X Reaction Buffer.

6 Initiate reaction by adding 100 µL from above to each well.

7 Place plate into Fluroskan holder and click **START.**

8 Take 4 readings at 15 minute intervals using 544/590 filter pairs.

9 Save raw data as an Excel file into the RHPx data folder. Use the naming convention RHXXXX.xls, where XXXX is the date in mmdd format.

10 Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.

11 Save organized data as an Excel file into the RHP data folder. Use the naming convention rhXXXXor.xls, where XXXX is the date in mmdd format.