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Antioxidant activity by reduced glutathione (GSH) assay: *in vitro* protocol

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ABSTRACT

Considering the role of oxidative stress in the pathology of several diseases and the use of antioxidants as treatment and/or adjuvants in these conditions. Here we propose a protocol to evaluate the antioxidant capacity of compounds by the GSH method, through the ability to prevent the oxidation of GSH (reduced glutathione) induced by hydrogen peroxide (H_2O_2), measured by the remaining groups of GSH that react with DTNB. This protocol was standardized at LAPCOM (Psychopharmacology and Behavior Laboratory at UFRGS) to assess biochemical parameters *in vitro*.

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MATERIALS TEXT

Monobasic potassium phosphate **NUCLEAR Catalog #318312** Step 1.1

Potassium phosphate dibasic **Neon Catalog #11361** Step 1.1

L-Glutathione reduced **Sigma Aldrich Catalog #G4251** Step 1.2

55'-Dithiobis(2-nitrobenzoic acid) **Sigma-aldrich Catalog #D8130** Step 1.3

Ethanol **Merck Millipore Catalog #100983** Step 1.3

Hydrogen peroxide 35% **Neon Catalog #0 1984** Step 2

Gloves **Contributed by users**

96 well plate **Contributed by users**

1.5 mL Eppendorf tubes **Contributed by users**

Surgical mask **Contributed by users**

Micropipette (0.5 - 10 µL) **Contributed by users**

Micropipette (100 - 1000 µL) **Contributed by users**

pH meter **Contributed by users**

Synergy™ HTX Multi-Mode Microplate Reader **Contributed by users**

Multichannel pipette (5 µL; 30- 300 µL) **Contributed by users**

Ultrapure water **Contributed by users**

SAFETY WARNINGS

Use personal protective equipment (including lab coat, masks, and gloves) whenever manipulating chemical and biological samples. Make sure to read all Safety Data Sheets for the reagents.

Preparing the reagents

1 The first step is to prepare the reagents to be used in this protocol;

1.1 Potassium phosphate buffer [M]1 Molarity (M) :

1.1.1 Weigh **13.609 g** of monobasic potassium phosphate (KH_2PO_4) in a beaker of appropriate size;

Monobasic potassium phosphate **NUCLEAR Catalog #318312**

1.1.2 Dissolve the salt with **90 mL** of ultrapure water;

1.1.3 Transfer the solution to a **100 mL** volumetric flask;

1.1.4 Using ultrapure water, complete the solution's volume to reach **100 mL** ;

1.1.5 Weigh **17.418 g** of dibasic potassium phosphate (K_2HPO_4) in a beaker of appropriate size;

Potassium phosphate dibasic **Neon Catalog #11361**

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1.1.6 Dissolve the salt with **90 mL** of ultrapure water;

1.1.7 Transfer the solution to a **100 mL** volumetric flask;

1.1.8 Mix both solutions slowly in a **500 mL** beaker following the steps below;

- Transfer **50 mL** of the monobasic potassium phosphate (KH_2PO_4) solution to the beaker;
- Use a pH sensor to evaluate your solution. Expected conditions: pH **6.8** ;

If the pH of your solution is lower than 6.8 adjust the pH adding drops of the dibasic potassium phosphate (K_2HPO_4) solution;

If the pH of your solution is above 6.8 adjust the pH adding drops of the monobasic potassium phosphate (KH_2PO_4) solution;

After adjusting the pH of this initial solution, proceed to add, slowly, drops of both buffer solutions (monobasic potassium phosphate and dibasic potassium phosphate). Use Pasteur pipettes to add the solutions. Mix your solutions using a pH sensor, making sure the mix of both buffers is always at pH **6.8** ;

1.2 Reduced glutathione (GSH) [M]60 Milimolar (mM) :

1.2.1 Weigh **0.02028 g** of GSH;

☒ L-Glutathione reduced **Sigma Aldrich Catalog #G4251**

1.2.2 Dissolve completely the GSH in **1.1 mL** of ultrapure water using a container of appropriate size;

1.2.3 Stock this solution at **-20 °C** , in samples of **220 µL** using plastic microtubes;

1.2.4 On the day of the *in vitro* test, remove one of the samples from the freezer;

1.2.5 Using a micropipette, transfer the content of the microtube (**220 µL**) to a test tube;

1.2.6 Wash the microtube used to store your sample one time with **980 µL** of ultrapure water and transfer this volume to the test tube;

1.2.7 Add **1000 µL** of ultrapure water to the test tube to obtain a solution of GSH **[M]6 Milimolar (mM)** ;

1.3 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) [M]10 Milimolar (mM) :

1.3.1 Weigh carefully **0.0396 g** of DTNB in a piece of aluminum foil;

☒ 55'-Dithiobis(2-nitrobenzoic acid) **Sigma-aldrich Catalog #D8130**

1.3.2 Transfer the DTNB to a beaker of appropriate size;

1.3.3 Add **9 mL** of absolute ethanol to the beaker to dissolve the salt;

☒ Ethanol **Merck Millipore Catalog #100983**

1.3.4 Transfer your solution to a **10 mL** volumetric flask;

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1.3.5 Using absolute ethanol, complete the solution's volume to reach **10 mL** ;

1.3.6 Store the solution in an amber flask of appropriate size covered with aluminum foil at **8 °C** ;

1.3.7 On the day of the *in vitro* test, remove the solution from the fridge;

1.3.8 Using a micropipette, transfer **1.25 mL** of the solution to a **100 mL** volumetric flask;

1.3.9 Using ultrapure water, complete the solution's volume to reach **100 mL** . You will obtain a solution of DTNB **0.125 Milimolar (mM)** ;

1.4 **Hydrogen peroxide (H_2O_2) solution:** Follow the steps described in section 2 of this protocol to first standardize and then prepare a hydrogen peroxide solution. This reagent should be prepared on the day of the biochemical assay, it should not be stored for later use;

Standardization of H202 solution

2 Every 30 days, or whenever a new bottle of hydrogen peroxide is opened, the absorbance of the concentrated solution should be checked.

Hydrogen peroxide

35% **Neon Catalog #0 1984**

2.1 First, we must calculate the molarity of the H_2O_2 solution in the bottle:

Concentration stated in the bottle: **35 % (m/v)** Molecular weight: **34.0147 g/mol**

1 M ---------- 1 mol ---------- 1000 mL
1 M ----- -----34.0147 g ------- 1000 mL
x ---------- 350 g ---------1000 mL

x = **10.29 Molarity (M)**

2.2 Prepare a **10 Milimolar (mM)** solution (**10 mL**) of H_2O_2:

2.2.1 Follow the calculations below to determine the volume of the primary solution needed:

C1 x V1 = C2 x V2

10.29 Molarity (M) x V1 = 0.01 Molarity (M) x 10 mL

V1 = 9.72 µL

2.2.2 Using a micropipette, collect **9.72 µl** of H_2O_2 and transfer to a **10 mL** volumetric flask;

2.2.3 Using ultrapure water, complete the solution's volume to reach **10 mL** ;

2.3 Read the absorbance of the solution at **240 nm** in a microplate reader;

2.4 Calculate the real concentration of your sample following these steps:

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A standardized [M]**10 Milimolar (mM)** H_2O_2 solution has an absorbance of **0.394**

10 mM H_2O_2 ---------- 0.394 Abs
X --------- **Absorbance of your sample**

X = Represents the real concentration of the hydrogen peroxide sample

2.5 Calculate the real concentration of the bottled solution following these steps:

As X (calculated on the last step) Represents the real concentration of the hydrogen peroxide sample

10 mM H_2O_2 ---------- 10.29 M
X --------- **Z**

Z = Real concentration of hydrogen peroxide in the bottle

2.6 Prepare a [M]**150 Milimolar (mM)** stock solution of H_2O_2: this reagent should be prepared on the day of the biochemical assay, it should not be stored for later use;

2.6.1 Follow the calculations below to determine the volume of the primary solution needed:

C1 x V1 = C2 x V2
Z (the real concentration determined on the last step) x V1 = [M]**0.15 Molarity (M)** **x** **10 mL**
V1 = depends on the concentration of your hydrogen peroxide solution

2.6.2 Using a micropipette, collect the determined volume of H_2O_2 and transfer it to a **10 mL** volumetric flask;

2.6.3 Using ultrapure water, complete the solution's volume to reach **10 mL** ;

2.6.4 Store this solution in an amber flask at **8 °C** when needed;

2.7 Prepare a [M]**5 Milimolar (mM)** solution from your [M]**150 Milimolar (mM)** stock solution:

2.7.1 Follow the calculations below to determine the volume of the stock solution needed:

C1 x V1 = C2 x V2
[M]**150 Milimolar (mM)** **x V1 =** [M]**5 Milimolar (mM)** **x** **10 mL**
V1 = **333.33 µL**

2.6.2 Using a micropipette, collect **333.33 µL** of the stock solution and transfer it to a **10 mL** volumetric flask;

2.6.3 Using ultrapure water, complete the solution's volume to reach **10 mL** ;

2.6.4 Store this solution in an amber flask at **8 °C** when needed;

Incubation of the samples 30m

3

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To optimize the reaction, an incubation step is needed.

3.1 Prepare **1.5 mL** microtubes, to be used to store the samples, with the correct information. Wrap the microtubes in aluminum foil. The number of microtubes depends on the number of samples. You should provide at least five replicates (n = 5) of each sample with at least one control tube per sample. You should also prepare negative and positive control samples for your GSH solution. Perform the test at least two times to ensure the results are as correct as possible.

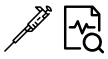
3.2 For each sample, fill the plastic microtubes as described below. Using a micropipette fill the tubes in this order: Potassium phosphate buffer + H_2O_2 + GSH + Sample. Mix the solution with the pipette tip to homogenize the content;

A	B	C	D	E
Microtubes	**Buffer (µL)**	**GSH (µL)**	**Sample (µL)**	**H2O2 (µL)**
Control blank	210	-	-	40
Sample blank	200	-	10	40
Control	170	40	-	40
Sample	160	40	10	40

30m

3.3 Incubate all your samples in the dark at **25 °C** for **00:30:00** ;

Reading your samples 5m

4 

Prepare to read the absorbance of your samples in a microplate reader;

4.1 Use a conventional 96-well microplate to run your samples. Before start pipetting, each well of the microplate should be marked for sample identification.

4.2 

Add **235 µL** of DTNB to all of the wells needed. Fill two additional wells with **250 µL** of DTNB to work as the negative control for the absorbance reading;

4.3 Transfer **15 µL** of the content of each microtube to its corresponding well in the microplate;

5m

4.4 Let your samples rest for **00:05:00** ;

4.5 Read the absorbance of the samples at **412 nm** in a microplate reader;

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Calculating data and determinig results

5 Prepare to analyze the results obtained after reading the absorbance of the samples;

5.1 Subtract the absorbance of the blank from the absorbance of the samples:

Sample absorbance = Sample - Sample blank

5.2 Calculate the mean absorbance of your replicates;

5.3 Determine the percentage of remaining sulfhydryls of GSH:

$$\%\ \text{of remaining sulfhydryls of GSH} = \left[\frac{Abs_{control} - (Abs_{sample} - Abs_{blank})}{Abs_{control}}\right] \times 100$$

$Abs_{control}$= The absorbance of control
Abs_{sample}= The absorbance of your sample - sample blank
Abs_{blank}= The absorbance of control blank

5.4 Results should be expressed as % of remaining sulfhydryls of GSH.

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