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# Antioxidant activity by Deoxyribose 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 different compounds by the TBARS method *in vitro* through the ability to inhibit the synthesis of hydroxyl radical from the oxidation of deoxyribose by hydrogen peroxide $H_2O_2$ via Fenton reaction. 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 hydroxide **Synth Catalog #H2002.01.AG** Step 1.1

2-Deoxy-D-ribose **Sigma-aldrich Catalog #31170** Step 1.2

Iron(III) chloride hexahydrate **Sigma-aldrich Catalog #236489** Step 1.3

Ethylenediaminetetraacetic acid **Sigma-aldrich Catalog #EDS** Step 1.4

Thiobarbituric acid (TBA) **J.T. Baker Catalog #V774-05** Step 1.6

Hydrochloric acid **Neon Catalog #2618** Step 1.7

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**

Compact Digital Dry Bath/ Block Heater Compact Dry Bath S 100-240V US plug **Thermo Scientific Catalog #88871001**

Ascorbic acid **REAGEN** Step 1.5

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 $KH_2PO_4$-KOH buffer [M]50 Milimolar (mM) :

1.1.1 Weigh **0.68043 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;



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

1.1.5 Weigh **0.280053 g** of potassium hydroxide (KOH) in a beaker of appropriate size;

Potassium hydroxide **Synth Catalog #H2002.01.AG**

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 **250 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 **7.4** ;

If the pH of your solution is lower than 7.4 adjust the pH adding drops of the potassium hydroxide (KOH) solution;

If the pH of your solution is above 7.4 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 potassium hydroxide). 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 **7.4** ;

1.1.9 Store this solution in an amber flask at **8 °C** ;

## 1.2 Deoxyribose [M]60 Milimolar (mM) :

1.2.1 Weigh **0.080478 g** of deoxyribose;

2-Deoxy-D-ribose **Sigma-aldrich Catalog #31170**

1.2.2 Transfer the deoxyribose to a beaker of appropriate size;

1.2.3 Add **9 mL** of ultrapure water to the beaker to dissolve the salt;

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

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

## 1.3 Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) [M]1 Milimolar (mM) :

1.3.1 Weigh **0.013515 g** of ferric chloride Iron(III) chloride hexahydrate;

Iron(III) chloride hexahydrate **Sigma-aldrich Catalog #236489**

1.3.2 Transfer the Iron(III) chloride hexahydrate to a beaker of appropriate size;

1.3.3 Add **40 mL** of ultrapure water to the beaker to dissolve the salt;

1.3.4 Transfer your solution to a **50 mL** volumetric flask;



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1.3.5 Using ultrapure water, complete the solution's volume to reach **50 mL** ;

## 1.4 Ethylenediaminetetraacetic acid (EDTA) [M]1.04 Milimolar (mM) :

1.4.1 Weigh **0.01519648 g** of EDTA;

Ethylenediaminetetraacetic acid **Sigma-aldrich Catalog #EDS**

1.4.2 Transfer the EDTA to a beaker of appropriate size;

1.4.3 Add **40 mL** of ultrapure water to the beaker to dissolve the salt;

1.4.4 Transfer your solution to a **50 mL** volumetric flask;

1.4.5 Using ultrapure water, complete the solution's volume to reach **50 mL** ;

## 1.5 Ascorbic acid [M]2 Milimolar (mM) :

1.5.1 Weigh **0.017612 g** of ascorbic acid;

Ascorbic acid **REAGEN**

1.5.2 Transfer the ascorbic acid to a beaker of appropriate size;

1.5.3 Add **40 mL** of ultrapure water to the beaker to dissolve the salt;

1.5.4 Transfer your solution to a **50 mL** volumetric flask;

1.5.5 Using ultrapure water, complete the solution's volume to reach **50 mL** ;

## 1.6 Thiobarbituric acid (TBA) [M]1 % :

1.6.1 Weigh **0.1 g** of TBA;

Thiobarbituric acid (TBA) **J.T. Baker Catalog #V774-05**

1.6.2 Transfer the ascorbic acid to a beaker of appropriate size;

1.6.3 Add **9 mL** of ultrapure water to the beaker to dissolve the salt;

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

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

## 1.7 Hydrochloric acid (HCl) [M]25 % :

1.7.1 Using a micropipette collect **6.757 mL** of HCl **[M]37 %** ;

Hydrochloric acid **Neon Catalog #2618**



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1.7.2 Transfer the HCl solution to a **10 mL** volumetric flask;

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

1.8 **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:

A standardized **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:



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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]**10 Milimolar (mM)** 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]0.010 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;

Incubation of the samples 1h 15m

3

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. 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. 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: $KH_2PO_4$–KOH buffer + Deoxyribose + $FeCl_3$ + EDTA + Ascorbic acid + $H_2O_2$ + Sample. Mix the solution with the pipette tip to homogenize the content;

| A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|
| **Microtubes** | **KH2PO4–KOH (µL)** | **Deoxyribose (µL)** | **FeCl3 (µL)** | **EDTA (µL** | **Ascorbic acid (µL)** | **H2O2 (µL)** | **Sample (µL)** |
| **Control** | 50 | 10 | 10 | 10 | 10 | 10 | - |
| **Sample blank** | 50 | - | 10 | 10 | 10 | 10 | 10 |
| **Sample** | 40 | 10 | 10 | 10 | 10 | 10 | 10 |



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1h

3.3 Incubate all your samples at **37 °C** for **01:00:00** using a dry bath;

3.4 Add to all of your microtubes **100 µL** of TBA **1 %** and **100 µL** of HCl **25 %** ;

15m

3.5 Incubate all your samples at **100 °C** for **00:15:00** using a dry bath;

## Reading your samples

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 Transfer **250 µL** of the content of each microtube to its corresponding well in the microplate;

4.3 Read the absorbance of the samples at **532 nm** in a microplate reader;

## Calculating data and determinig results

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

5.1 Calculate the mean absorbance of your replicates;

5.2 Determine the percentage of inhibition of the hydroxyl radical:

$$\%\ \text{of inhibition of the hydroxyl radical} = \left[\frac{Abs_{control} - (Abs_{sample} - Abs_{blank})}{Abs_{control}}\right] \times 100$$

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



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5.3 Results should be expressed as % of inhibition of the hydroxyl radical.



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