Jun 15, 2021

Antioxidant activity by FRAP assay: *in vitro* protocol

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dx.doi.org/10.17504/protocols.io.btqrnmv6

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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 FRAP method, which is based on the reduction of Fe3+ (ferric) to Fe2+ (ferrous) by antioxidant molecules forming the blue-colored Fe2++ TPTZ complex. This protocol was standardized at LAPCOM (Psychopharmacology and Behavior Laboratory at UFRGS) to assess biochemical parameters *in vitro.*

DOI

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PROTOCOL CITATION

Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Ana P Herrmann, Angelo Piato 2021. Antioxidant activity by FRAP assay: in vitro protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btqrnmv6

CREATED

Mar 28, 2021

LAST MODIFIED

Jun 15, 2021

OWNERSHIP HISTORY

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Jun 15, 2021 Angelo Piato Universidade Federal do Rio Grande do Sul

PROTOCOL INTEGER ID

48625

MATERIALS TEXT

Sodium Acetate Anhydrous **Sigma-aldrich Catalog #W302406** Step 1.1

Acetic acid **Sigma-aldrich Catalog #A6283** Step 1.1

246-Tris(2-pyridyl)-s-triazine **Sigma-aldrich Catalog #T1253** Step 1.2

Hydrochloric acid **Neon Catalog #2618** Step 1.2

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

Iron(II) sulfate heptahydrate **Sigma-aldrich Catalog #215422** Step 1.4

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**

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 **Acetate buffer [M]300 mmol/L :**

1.1.1 Weigh **0.19 g** of sodium acetate in a beaker of appropriate size;

Sodium Acetate Anhydrous **Sigma-aldrich Catalog #W302406**

1.1.2 Dissolve the salt with **10 mL** of ultrapure water;

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

1.1.4 Add slowly **1.6 mL** of glacial acetic acid to the volumetric flask;

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Acetic acid **Sigma-aldrich Catalog #A6283**

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

1.1.6 Use a pH sensor to evaluate your solution. Expected conditions: pH **3.6** ;

- If the pH of your solution is above 3.6 adjust the pH by adding drops of glacial acetic acid;

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

1.2 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) [M] 10 Milimolar (mM) + Hydrochloric acid (HCl) [M] 40 Milimolar (mM) :

1.2.1 Pepare a solution of HCl [M] **40 Milimolar (mM)** using HCl [M] **37 %** ;

Hydrochloric acid **Neon Catalog #2618**

1.2.2 Fill a **10 mL** volumetric flask with **5 mL** of ultrapure water;

1.2.3 Add **40 µl** of HCl [M] **37 %** to the volumetric flask;

1.2.4 Using ultrapure water, complete the solution's volume to reach **10 mL** . (This solution is suited for use for 2 days);

1.2.5 Weigh **0.031 g** of TPTZ in a beaker of appropriate size;

246-Tris(2-pyridyl)-s-triazine **Sigma-aldrich Catalog #T1253**

1.2.6 Add **8 mL** of HCl [M] **40 Milimolar (mM)** ;

1.2.8 Transfer the solution to a **10 mL** volumetric flask;

1.2.8 Using HCl [M] **40 Milimolar (mM)** , complete the solution's volume to reach **10 mL** ;

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

1.3 Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) [M] 20 Milimolar (mM) :

1.3.1 Weigh **0.0541 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 **5 mL** of ultrapure water to the beaker to dissolve the salt;

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

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

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1.3.6 Prepare this solution on the day of the experiment;

1.4 Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$) [M]2000 Micromolar (µM) :

1.4.1 Weigh **0.0556 g** of Iron(II) sulfate heptahydrate;

Iron(II) sulfate heptahydrate **Sigma-aldrich Catalog #215422**

1.4.2 Transfer the Iron(II) sulfate heptahydrate to a beaker of appropriate size;

1.4.3 Add **90 mL** of ultrapure water to the beaker to dissolve the salt;

1.4.4 Transfer your solution to a **100 mL** volumetric flask;

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

1.4.6 Prepare this solution on the day of the experiment;

Working solutions

2 ⚠

These solutions should be prepared only on the day of the assay by mixing the previously described reagents. You should mix the solutions in a container of appropriate size wrapped in aluminum foil. Keep the solutions on ice.

2.1 Working solution without TPTZ (perform this step only if the sample has color):

Mix the following reagents in a proportion of 10:1:1

- Acetate buffer **15 mL** ;
- Ultrapure water **1.5 mL** ;
- Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) **1.5 mL** ;

2.2 Working solution with TPTZ:

Mix the following reagents in a proportion of 10:1:1

- Acetate buffer **15 mL** ;
- TPTZ solution **1.5 mL** ;
- Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) **1.5 mL** ;

Incubation of the samples 20m

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 To generate the standard curve, fill the wells of your microplate as described below. You should provide

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duplicates or triplicates of each point of the curve to make your quantification more precise. Using a micropipette fill the wells in this order: Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$) + Ultrapure water + Working solution with TPTZ. Air bubbles should be perforated with a needle to avoid bias in the analysis;

A	B	C	D
Microtube concentration of FeSO4.7H2O	**FeSO4.7H2O (µL)**	**Ultrapure water (µL)**	**Working solution with TPTZ (µL)**
0	-	10	300
100	0.5	9.5	300
400	2	8	300
800	4	6	300
1200	6	4	300
1600	8	2	300
2000	10	-	300

3.3 For each sample, fill the plastic microtubes as described below. Using a micropipette fill the tubes in this order: Sample + Ultrapure water + Working reagent without TPTZ or Working reagent with TPTZ. Mix the solution with the pipette tip to homogenize the content;

A	B	C	D	E
Microtube	**Sample (µL)**	**Ultrapure water (µL)**	**Working reagent without TPTZ (µL)**	**Working reagent with TPTZ (µL)**
Blank	-	10	-	300
Sample blank	10	-	300	-
Sample	10	-	-	300

3.4 Incubate all your microtubes at **37 °C** for **00:15:00** using a dry bath; 15m

3.5 Take your samples off the dry bath and let them rest at room temperature for **00:05:00** ; 5m

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;

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4.3 Read the absorbance of the samples at **593 nm** in a microplate reader;

Calculating data and determinig results

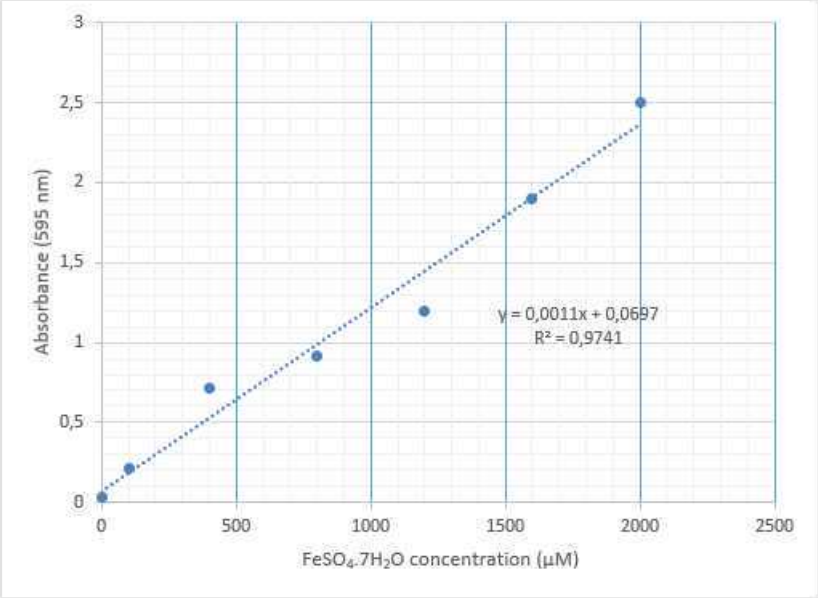
5

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

5.1 Subtract the absorbance of the blank wells from the absorbance of the different $FeSO_4.7H_2O$ concentrations:

Absorbance of $FeSO_4.7H_2O$ = Absorbance of $FeSO_4.7H_2O$ - Blank absorbance

5.2 Plot in an excel sheet the results from your standard curve. Ferrous sulfate ($FeSO_4.7H_2O$) concentrations in µM are plotted on the X axis, while the respective absorbances (Sample absorbance - Blank absorbance) Should be plotted on the Y axis (as shown in the figure below). Insert a scatter plot, add a trend line, line equation, and R^2 value. The value of R^2 must be between 0.9 and 1.



5.3 Subtract the absorbance of the sample blank wells from the absorbance of the different samples:

Sample absorbance = Sample absorbance - Sample blank absorbance

5.4 From the line equation, replace the Y with the sample absorbance (Sample absorbance - Sample blank) and calculate the X value, resulting in Fe^{2+} concentration in micromoles per liter (µM) in the analyzed sample.

5.5 Calculate the amount of Fe^{2+} in micromoles (µmol) in the final volume of the microtube:

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Microtube volume: 0.31 mL

Fe^{2+} concentration found in the lat step ---------- 1000 mL
Y ---------- 0.31 mL
Y = ... equivalent µmoles of Fe^{2+}

5.6 Calculate the amount of Fe^{2+} per mL of sample used in the technique:

Y found in the last step ---------- 0.01 mL
Z ---------- 1 mL
Z = ... equivalent µmoles of Fe^{2+} / mL of sample

5.7 Results should be expressed as equivalent µmoles of Fe^{2+}/mL of the sample.

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