Jun 15, 2021

Glutathione peroxidase (GPx) activity assessment for zebrafish brain tissue

Adrieli Sachett[1], Matheus Gallas-Lopes[1], Greicy M M Conterato[2], Ana P Herrmann[1], Angelo Piato[1]

[1]Universidade Federal do Rio Grande do Sul; [2]Universidade Federal de Santa Catarina

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


Angelo Piato
Universidade Federal do Rio Grande do Sul

ABSTRACT

Zebrafish are being increasingly used as a model animal in neuroscience research. Here we describe a protocol to assess brain tissue activity of glutathione peroxidase (GPx), an enzyme glutathione family, and act how the main remover of hydrogen peroxide in zebrafish brain and whose main biological role is to protect the organism from oxidative damage.

DOI

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

PROTOCOL CITATION

Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Ana P Herrmann, Angelo Piato 2021. Glutathione peroxidase (GPx) activity assessment for zebrafish brain tissue. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bsujneun

CREATED

Feb 27, 2021

LAST MODIFIED

Jun 15, 2021

OWNERSHIP HISTORY

Feb 27, 2021 Matheus Gallas-Lopes Universidade Federal do Rio Grande do Sul

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

PROTOCOL INTEGER ID

47723

GUIDELINES

This protocol is intended to standardize glutathione peroxidase activity assessment in zebrafish brain tissue samples. It can be adapted for other fish species.

MATERIALS TEXT

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**

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

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

Ethylenediaminetetraacetic acid disodium salt dihydrate **Sigma Aldrich Catalog #E5134-1KG** Step 1.2

L-Glutathione reduced **Sigma Aldrich Catalog #G4251** Step 1.3

NADPH Tetrasodium salt **Sigma Aldrich Catalog #10107824001** Step 1.4

Sodium azide **Sigma Aldrich Catalog #S2002** Step 1.5

Glutathione Reductase from bakers yeast (S. cerevisiae) **Sigma Aldrich Catalog #G3664** Step 1.6

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

SAFETY WARNINGS

Use personal protective equipment (including lab coats, masks, and gloves) when manipulating chemical and biological samples. Read the Safety Data Sheets of the reagents.

BEFORE STARTING

This protocol was standardized at LAPCOM (Psychopharmacology and Behavior Laboratory at UFRGS) to assess biochemical parameters in zebrafish brain tissue. Protocols you should read before proceeding with this method:

Adrieli Sachett, Matheus Gallas-Lopes, Radharani , Greicy M M Conterato, Ana Herrmann, Angelo Piato. How to prepare zebrafish brain tissue samples for biochemical assays.
http://dx.doi.org/10.17504/protocols.io.bjkdkks6

Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani , Ana Herrmann, Angelo Piato. Protein quantification protocol optimized for zebrafish brain tissue (Bradford method).
http://dx.doi.org/10.17504/protocols.io.bjnfkmbn

Citation: Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Ana P Herrmann, Angelo Piato (06/15/2021). Glutathione peroxidase (GPx) activity assessment for zebrafish brain tissue. https://dx.doi.org/10.17504/protocols.io.bsujneun

Preparing the reagents

1 The first step is to prepare the reagents to be used in the assessment of glutathione peroxidase activity;

1.1 Potassium phosphate buffer [M]0.5 Molarity (M) :

1.1.1 Weigh **6.804 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 **8.709 g** of dibasic potassium phosphate (K_2HPO_4) in a beaker of appropriate size;

Potassium phosphate dibasic **Neon Catalog #11361**

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 **7.0** ;

If the pH of your solution is lower than 7 adjust the pH adding drops of the dibasic potassium phosphate (K_2HPO_4) solution;

If the pH of your solution is above 7 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 **7.0** ;

1.2 EDTA (Ethylenediaminetetraacetic acid) [M]5 Milimolar (mM) + Potassium phosphate buffer:

1.2.1 Weigh **0.186 g** of EDTA;

Ethylenediaminetetraacetic acid disodium salt dihydrate **Sigma Aldrich Catalog #E5134-1KG**

1.2.2 Dissolve completely the EDTA powder in **90 mL** of the potassium phosphate buffer solution

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prepared in the last step using a beaker of appropriate size;

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

1.2.4 Using the potassium phosphate buffer solution prepared in the last step, complete the solution's volume to reach **100 mL** ;

1.2.5 Use a pH sensor to evaluate your solution. Expected conditions: pH **7.0** ;

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

1.3 Reduced glutathione (GSH) [M]10 Milimolar (mM) :

1.3.1 Weigh **0.0154 g** of GSH;

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

1.3.2 Dissolve completely the GSH in **5 mL** of ultrapure water using a container of appropriate size;

1.3.3 Stock this solution at **-20 °C** , in samples of **500 µL** using plastic microtubes;

1.4 NADPH (Dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt) [M]1.6 Milimolar (mM) :

1.4.1 Weigh **0.013 g** of NADPH;

NADPH Tetrasodium salt **Sigma Aldrich Catalog #10107824001**

1.4.2 Dissolve completely the NADPH in **10 mL** of ultrapure water using a container of appropriate size;

1.4.3 Stock this solution at **-20 °C** , in samples of **500 µL** using plastic microtubes;

1.5 Sodium azide (NaN_3) [M]10 Milimolar (mM) :

1.5.1 Weigh **0.0065 g** of sodium azide;

Sodium azide **Sigma Aldrich Catalog #S2002**

1.5.2 Dissolve completely the sodium azide powder in **10 mL** of ultrapure water using a container of appropriate size;

1.5.3 Store the solution at room temperature;

1.6

Glutathione reductase (GR) [M]0.5 U/mL : this reagent should be prepared on the day of the biochemical assay, it should not be stored for later use;

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1.6.1 Each batch of the enzyme has a specific concentration, so every time you open a new flask you should redo the calculations as follows to prepare the solution:

Liquid GR: [M]1.3 mg protein/mL or **[M]214 U/mg protein:**

☒Glutathione Reductase from bakers yeast (S. cerevisiae) **Sigma Aldrich Catalog #G3664**

214 U – 1 mg protein
X – 1.3 mg protein
X = 278.2 U

278.2 U – 1 mL
0.5 U – X
X = 0.001797 mL = 0.002 mL = 2 µL

1.6.2 Using a micropipette, fill a plastic microtube with **998 µL** of ultrapure water;

1.6.3 Using a micropipette, collect **2 µL** of the liquid GR and transfer it to the microtube containing ultrapure water, homogenizing the solution;

1.7 **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: **[M]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 = **[M]10.29 Molarity (M)**

2.2 Prepare a **[M]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
[M]10.29 Molarity (M) x V1 = [M]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;

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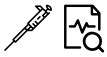
2.2.3 Using ultrapure water, complete the solution's volume to reach **10 mL** ;

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:

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 **1 Molarity (M)** 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 = 1 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 **4 Milimolar (mM)** solution from your **1 Molarity (M)** stock solution:

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

C1 x V1 = C2 x V2
1 Molarity (M) x V1 = 0.004 Molarity (M) x 10 mL
V1 = 40 µL

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2.6.2 Using a micropipette, collect **40 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;

Microplate preparation and absorbance reading 10m 5s

3 ⚠

Use a conventional 96-well microplate to run your samples. Tissue sample collection and preparation are described elsewhere;

> Adrieli Sachett, Matheus Gallas-Lopes, Radharani , Greicy M M Conterato, Ana Herrmann, Angelo Piato. How to prepare zebrafish brain tissue samples for biochemical assays.
> http://dx.doi.org/10.17504/protocols.io.bjkdkks6

3.1 You must calculate the sample volume that corresponds to **30 µg** of proteins. This calculation is based on the Bradford method described elsewhere;

> Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani , Ana Herrmann, Angelo Piato. Protein quantification protocol optimized for zebrafish brain tissue (Bradford method).
> http://dx.doi.org/10.17504/protocols.io.bjnfkmbn

3.1.1 To estimate the volume of the sample corresponding to **30 µg** of proteins, divide the amount of protein needed (**30 µg**) by the total amount of proteins in the sample quantified by the Bradford method (example below);

Volume of the sample needed for the assay (µL) = 30 µg / total amount of proteins in the sample µg/µL

3.2 Place and keep your Potassium phosphate buffer/EDTA **go to step #1.2** on a water bath at **30 °C** throughout the analysis;

3.3 5m 15s

Set your microplate reader to the following configuration: Kinetic mode with agitation; **340 nm** ;
Readings every **00:00:15** for **00:05:00** ; **30 °C** ;

3.4 Using an adequate micropipette, fill the wells of your microplate as described below. You should provide duplicates or triplicates of each sample as stated above. Using a micropipette fill the wells in this order: Tissue sample, Potassium phosphate buffer/EDTA, GSH, NADPH, NaN_3, and GR. Lastly, you

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must add the H_2O_2 solution **as quickly as possible** using a multichannel pipette while being careful to homogenize the content of the wells. The Potassium phosphate buffer/EDTA volume depends on the volume of the sample. All wells should have a final volume of **250 µL** , so the Potassium phosphate buffer/EDTA is used so that every solution reaches this volume (e.g., **50 µL** of the sample + **75 µL** of the Potassium phosphate buffer/EDTA + **25 µL** of GSH + **25 µL** of NADPH + **25 µL** NaN_3 + **25 µL** of GR + **25 µL** of H_2O_2);

A	B	C	D	E	F	G	H
Well	**Sample (µL)**	**Potassium phosphate buffer/EDTA 0.5 M (µL)**	**GSH 10 mM (µL)**	**NADPH 1.6 mM (µL)**	**NaN3 10 mM (µL)**	**GR 0.5 U/mL (µL)**	**H2O2 (µL)**
Control	0	125	25	25	25	25	25
Samples	Depends on the volume of the sample corresponding to 30 µg of proteins.	Depends on the volume of the sample. Volume needed for the final solution in the tube to reach 250 µL. (125 - sample volume)	25	25	25	25	25

3.5 Read the absorbance of the samples at the set conditions. You should see the absorbance of the sample wells decrease throughout the **00:05:00** of measurement; 5m

Calculating data and determinig results 8m

4 Choose the best **00:01:00** interval within the **00:05:00** of sampling. To do so check for the interval where the decrease between absorbances is the steepest; 6m

4.1 Calculate the mean absorbance of the wells containing the control solution and the wells containing your samples for each reading. Take into consideration whether you chose to use duplicates or triplicates of your samples.

4.2 Calculate the difference between the mean absorbance of the control samples within the **00:01:00** interval that was chosen; 1m

Δcontrol = (Mean absorbance at 0 s - Mean absorbance after 60 s)

4.3 Calculate the difference between the mean absorbance of each of your samples within the **00:01:00** interval that was chosen; 1m

Δsample = (Mean absorbance at 0 s - Mean absorbance at 60 s)

4.4 Subtract the Δcontrol value from the Δsample value for each of the samples;

Absorbance of the sample (Δ min) = (Δsample - Δcontrol)

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4.5 Determine GPx activity:

GPx activity = (Δ min x final volume (mL) x 1000000 x 1 mg/(6.22 x 1000 mL x amount of protein (mg))

GPx activity = (Δ min x 0,25 mL x 1000000 x 1 mg/(6.22 x 1000 mL x 0,03 mg)

Simplifying: GPx activity = (Δ min x 250000)/186.6

P.S.: 6.22 mM^{-1} cm^{-1} = molar extinction coefficients of NADPH

4.6 Final results are expressed as nmol NADPH/min/mg of protein.

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