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Quantification of nonprotein sulfhydryl groups (NPSH) optimized for zebrafish brain tissue

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bjrkkm4w

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ABSTRACT

Zebrafish are incresingly used as a model animal in neuroscience research. Here we describe a protocol to quantify nonprotein sulfhydryl groups (NPSH), an indirect evaluation of the levels of reduced glutathione (GSH), a major oxidative stress defense in the central nervous system.

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KEYWORDS

NPSH, Oxidative stress, Zebrafish brain tissue

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GUIDELINES

This protocol is intended to standardize nonprotein sulfhydryl groups quantification of zebrafish brain tissue samples. It can be adapted for other fish species.

MATERIALS

NAME	CATALOG #	VENDOR
Gloves		
96 well plate		
Eppendorf tubes 1.5 mL uncolored	022363204	Eppendorf Centrifuge
MiniV ortexer	58816-121	VWR Scientific
Surgical mask		
Micropipette (0.5 - 10 µL)		
Micropipette (100 - 1000 µL)		
pH meter		
Centrifuge 5424 R	5404000022	Eppendorf
Synergy™ HTX Multi-Mode Microplate Reader		Biotek

STEPS MATERIALS

NAME	CATALOG #	VENDOR
55'-Dithiobis(2-nitrobenzoic acid)	D8130	Sigma-aldrich
Ethanol	100983	Merck Millipore
Monobasic potssium phosphate	318312	NUCLEAR
Potassium phosphate dibasic	11361	Neon
Trichloroacetic acid (TCA)	T6399	Sigma – Aldrich

SAFETY WARNINGS

Use personal protective equipment (including lab coat, 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 Benvenutti, Greicy M M Conterato, Ana Herrmann, Angelo Piato. How to prepare zebrafish brain tissue samples for biochemical assays.
https://protocols.io/view/how-to-prepare-zebrafish-brain-tissue-samples-for-bjkdkks6

Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani Benvenutti, Ana Herrmann, Angelo Piato. Optimized protein quantification protocol for zebrafish brain tissue (Bradford method).
https://protocols.io/view/optimized-protein-quantification-protocol-for-zebr-bjnfkmbn

Preparing the reagents

1 The first step is to prepare the reagents to be used in the quantification of nonprotein sulfhydryl groups (NPSH);

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1.1 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) [M]10 mM :

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

55'-Dithiobis(2-nitrobenzoic acid)
by Sigma-aldrich
Catalog #: D8130

1.1.2 Transfer the DTNB to a beaker of appropriate size;

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

Ethanol
by Merck Millipore
Catalog #: 100983

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

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

Ethanol
by Merck Millipore
Catalog #: 100983

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

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

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

Monobasic potssium phosphate
by NUCLEAR
Catalog #: 318312
CAS Number: 7778-77-0

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

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

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

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

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Potassium phosphate dibasic
by Neon
Catalog #: 11361

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

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

1.2.8 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** ;

1.3 Trichloroacetic acid (TCA) 6%:

1.3.1 Weigh **6 g** of TCA in a beaker of an appropriate size;

Trichloroacetic acid (TCA)
by Sigma – Aldrich
Catalog #: T6399

1.3.2 Dissolve the TCA with **50 mL** of ultrapure water;

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

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

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

Deproteinizaton of your samples

2 To proceed with the quantification of nonprotein sulfhydryl groups in your samples, you first have to deproteinize them following the steps below. Tissue sample collection and preparation are described elsewhere;

Citation: Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani Benvenutti , Ana P Herrmann, Angelo Piato (09/08/2020). Quantification of nonprotein sulfhydryl groups (NPSH) optimized for zebrafish brain tissue. https://dx.doi.org/10.17504/protocols.io.bjrkkm4w

Adrieli Sachett, Matheus Gallas-Lopes, Radharani Benvenutti, Greicy M M Conterato, Ana Herrmann, Angelo Piato. How to prepare zebrafish brain tissue samples for biochemical assays.
https://protocols.io/view/how-to-prepare-zebrafish-brain-tissue-samples-for-bjkdkks6

2.1 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;

2.2 Before preparing your samples for deproteinization, you must calculate the sample volume that corresponds to **50 µg** of proteins. This calculation can be based on the Bradford method described elsewhere;

Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani Benvenutti, Ana Herrmann, Angelo Piato. Optimized protein quantification protocol for zebrafish brain tissue (Bradford method).
https://protocols.io/view/optimized-protein-quantification-protocol-for-zebr-bjnfkmbn

2.2.1 To estimate the volume of the sample corresponding to **50 µg** of proteins, divide the amount of protein needed (**50 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) = 50 µg / total amount of proteins in the sample µg/µL

2.3 For each tissue sample, fill the plastic microtubes as described below. You should provide duplicates or triplicates of each sample to make your quantification more precise. The sample volume corresponding to **50 µg** of proteins is the volume needed to fill one of the wells of the microplate, so if you are planning to evaluate your samples in duplicates fill the microtube with two times that volume, if evaluating in triplicates, three times that volume, and so on. Using a micropipette fill the tubes in this order: Sample and TCA solution (mixing the solution with the pipette tip to homogenize the content);

Sample (µL)	TCA 6% (µL)

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Depends on the volume of the sample corresponding to 50 µg of proteins and the number of replicates of the same sample you are planning to evaluate	Depends on the volume of the sample. You should add the same volume of TCA solution as the volume of the sample you are planning to use (1:1)

2.4 Use a vortexer to mix the samples for **00:00:10** ;

2.5 

Centrifuge the samples **10000 x g, 4°C, 00:05:00** ;

Microplate preparation and absorbance reading

3 

Use a conventional 96-well microplate to run your samples. Reagents should be at room temperature. Pipetting of DTNB should be performed under dim or no light, making sure the microplate is carefully covered in aluminum foil to avoid photodegradation of the reagent;

3.1 Before start pipetting, each well of the microplate should be marked for sample identification;

3.2 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: sample and TFK. The TFK volume depends on the volume of the sample. All wells should have a final volume of **245 µL** , so the TFK is used so that every solution reaches this volume (e.g., **50 µL** of the sample + **195 µL** of the TFK solution);

Well of the plate	Sample (µL)	TFK 1 M (µL)
Control	0	245
Samples	Depends on the volume of the sample corresponding to 50 µg of proteins.	Depends on the volume of the sample. Volume needed for the final solution in the well to reach 245 µL.

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

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3.4 After reading the absorbance of the samples, add, in the dark, **15 µL** of the DTNB solution **10 mM** to each well (control and samples) previously filled (mixing the solution with the pipette tip to homogenize the content of wells). The final volume of every well should reach **260 µL** ;

3.5 

Leave your microplate in a dark room to incubate at room temperature for **01:00:00** ;

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

Calculating data and determinig results

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

4.1 Calculate the mean absorbance of the control solution both before and after adding the DTNB;

Δcontrol = (Mean absorbance after DTNB - Mean absorbance before DTNB)

4.2 Calculate the mean absorbance of the samples both before and after adding the DTNB;

Δsample = (Mean absorbance after DTNB - Mean absorbance before DTNB)

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

Absorbance of the sample = (Δsample - Δcontrol)

4.4 Determine the number of moles of the group sulfhydryl (SH) in the sample using the calculation below;

$$\text{Moles of SH} = \frac{Abs_{sample} \times 0.00021}{14.15 \times 0.67}$$

4.5 Results should be expressed as µmol of SH groups;

µmol SH = Moles SH x 1000000

4.6 Calculate the amount of sulfhydryl groups per milligrams of protein;

$$\text{µmol SH/mg protein} = \frac{\text{µmol SH} \times 1000}{\text{(amount of proteins in the sample [50 µg in this case])}}$$

4.7 Final results are expressed as µmol SH/mg protein.

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