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Quantification of thiobarbituric acid reactive species (TBARS) optimized for zebrafish brain tissue

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1 Works for me dx.doi.org/10.17504/protocols.io.bjp8kmrw

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ABSTRACT

Zebrafish are increasingly used as a model animal in neuroscience research. Here we describe our protocol to quantify thiobarbituric acid reactive species (TBARS) in zebrafish brain tissue. TBARS levels are indicative of lipid peroxidation.

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KEYWORDS

TBARS, Oxidative stress, Zebrafish brain tissue

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GUIDELINES

This protocol is intended to standardize quantification of thiobarbituric acid reactive species in zebrafish brain tissue samples. It can be adapted for other fish species.

MATERIALS

NAME	CATALOG #	VENDOR
Gloves		
Incubator		
96 well plate		
Eppendorf tubes 1.5 mL uncolored	022363204	Eppendorf Centrifuge
Compact Digital Dry Bath/ Block Heater, Compact Dry Bath S, 100-240V, US plug	88871001	Thermo Fisher
Surgical mask		
Micropipette (0.5 - 10 µL)		
Micropipette (100 - 1000 µL)		
Multichannel pipette (5 µL; 30-300 µL)		
Adhesive tape		
Synergy™ HTX Multi-Mode Microplate Reader		Biotek

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Trichloroacetic acid (TCA)	T6399	Sigma – Aldrich
Thiobarbituric acid (TBA)		J.T. Baker
Malondialdehyde tetrabutylammonium salt	36357	Sigma-aldrich
Ethanol	100983	Merck Millipore

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.

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 thiobarbituric acid reactive species (TBARS) in the samples;

Citation: Adrieli Sachett, Matheus Gallas-Lopes, Greicy M M Conterato, Radharani Benvenutti , Ana P Herrmann, Angelo Piato (09/08/2020). Quantification ofÃÂ thiobarbituric acid reactive species (TBARS) optimized for zebrafish brain tissue. https://dx.doi.org/10.17504/protocols.io.bjp8kmrw

1.1 **Trichloroacetic acid (TCA) 20% + Thiobarbituric acid (TBA) 0.5%:** this reagent should be prepared on the day of the biochemical assay, it should not be stored for later use;

1.1.1 Weigh **2 g** of TCA in a beaker of appropriate size;

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

1.1.2 Dissolve the TCA with **8 mL** of ultrapure water;

1.1.3 Weigh carefully **0.05 g** of TBA in a piece of aluminum foil;

Thiobarbituric acid (TBA)
by J.T. Baker

1.1.4 Add the TBA to the solution of water + TCA. Use a heating plate to help dissolve the TBA in the solution;

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

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

1.2 **Malondialdehyde (MDA):** This reagent is volatile, be careful to maintain the storing flask closed;

Preparing a stock solution [M]2000 nmol/mL :

1.2.1 Weigh carefully **0.00627 g** of MDA in a piece of aluminum foil;

Malondialdehyde tetrabutylammonium salt
by Sigma-aldrich
Catalog #: 36357
CAS Number: 100683-54-3

1.2.2 Transfer the MDA to a beaker of appropriate size;

1.2.3 Add, slowly, **10 mL** of absolute ethanol to the beaker to dissolve the salt;

Ethanol
by Merck Millipore
Catalog #: 100983

1.2.4 Store the solution in an amber flask of appropriate size at **8 °C** ;

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1.2.5 Each time you proceed with this thiobarbituric acid reactive species quantification method, prepare a **[M]20 nmol/mL** sample solution from your stock solution following the calculation below;

C1 x V1 = C2 x V2

[M]2000 nmol/mL x V1 = [M]20 nmol/mL x 5 mL

V1 = 0.05 mL of the stock solution (50 µL)

1.2.6 Using a micropipette, collect **50 µL** of the stock solution and mix it to **4950 µL** of absolute ethanol;

Ethanol
by Merck Millipore
Catalog #: 100983

1.2.7 This diluted solution should be prepared on the day of the biochemical assay, it should not be stored for later use;

Incubation of the samples + standard curve

2 

To optimize the reaction that forms the thiobarbituric acid reactive species, an incubation step is needed. Tissue sample collection and preparation are described elsewhere;

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** heat resistant microtubes, to be used to store the samples, with the correct information. The number of microtubes depends on the number of samples and points of the standard curve;

2.2 For each point of the standard curve, fill the plastic microtubes as described below. You should provide duplicates or triplicates of each point of the curve to make your quantification more precise. Using a micropipette fill the tubes in this order: MDA solution, ultrapure water, and TCA + TBA solution (mixing the solution with the pipette tip to homogenize the content);

Point of the curve	MDA (20 nmol/mL) (µL)	Ultrapure water (µL)	TCA 2% + TBA 0.05% solution (µL)
Control (0 nmol of MDA)	**0**	**100**	**150**
Point 1 (0.4 nmol of MDA)	20	80	150

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Point 2 (0.8 nmol of MDA)	40	60	150
Point 3 (1.2 nmol of MDA)	60	40	150
Point 4 (1.6 nmol of MDA)	80	20	150
Point 5 (2.0 nmol of MDA)	100	0	150

The final volume in all microtubes should be 250 µL.

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

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2.3.1 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

2.4 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. Using a micropipette fill the tubes in this order: sample, ultrapure water, and TCA + TBA solution (mixing the solution with the pipette tip to homogenize the content). The water volume depends on the volume of the sample. All microtubes should have a final volume of **250 µL** , so water is used so that every solution reaches this volume (e.g. **50 µL** of the sample + **150 µL** of the TCA + TBA solution + **50 µL** of water);

Microtubes	Sample (µL)	Ultrapure water (µL)	TCA 2% + TBA 0.05% solution (µL)
Control	0	100	150
Sample	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 tube to reach 250 µL.	150

2.5 All microtubes should be correctly closed and sealed with adhesive tape to avoid that the microtubes open during the incubation period;

2.6 Incubate all your samples at **100 °C** for **00:30:00** using a dry bath;

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Reading your samples

3

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

3.1 Use a conventional 96-well microplate to run your samples. Before start pipetting, each well of the microplate should be marked for sample identification.. Standard curve samples and tissue samples can be read on the same plate. Transfer **200 µL** of the content of each microtube to it's corresponding well in the microplate;

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

Calculating data and determinig results

4 The calculations are based on the MDA curve (similar to the calculation of the protein curve described in other protocols);

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4.1 Calculate the correction factor for your MDA standard curve;

4.1.1 Correction factor (CF): Subtract the absorbance value of the control point of the curve from the mean absorbance of the point you are calculating. Divide the concentration of MDA of the well/point of the curve by the resulting value from the subtraction before;

$$CF = \frac{[\ ]\ MDA\ (nmol)}{Mean\ Abs - Control\ Abs}$$

4.1.2 Mean correction factor (MCF): The mean correction factor is calculated by the arithmetic mean of the correction factors for each point of the curve;

$$MCF = \sum Correction\ factors\ /\ Count\ of\ correction\ factors$$

or

$$MCF = \frac{F_{C1} + F_{C2} + F_{C3} + F_{C4} + F_{C5}}{5_{(number\ of\ factors)}}$$

4.2 The amount of thiobarbituric acid reactive species in your samples is calculated by multiplying the corrected mean absorbance of your sample to the mean correction factor calculated above;

TBARS = (Mean absorbance of the sample - Mean absorbance of the control sample) x MCF

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4.3 Results should be expressed as nmol of TBARS/mg of protein. To do so, divide the result obtained above for the amount of thiobarbituric acid reactive species in your sample by the amount of protein that you used of your sample (**50 µg** or **0.05 mg** in this protocol).

nmol TBARS/mg protein = nmol TBARS on the sample / 0.05 mg of proteins

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