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Optimized protocol for quantification of nitrite levels in brain and head kidney tissue samples in adult zebrafish

Forked from Optimized protocol for brain and head kidney catalase activity in zebrafish

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Fish behavior and physi...

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Protocol status: Working
We use this protocol and it's working

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Abstract

Zebrafish, and other small teleosts, are used as experimental models to evaluate human pathologies, including those linked to oxidative and nitrosative stress, inflammation, and nitric oxide signaling. The protocol presents an optimized technique to quantify nitrite levels, in zebrafish tissues, focusing on the brain and head kidney. The protocol is based on the classical Griess diazotization reaction (Griess, 1858) method.

Guidelines

This protocol is intended to quantify nitrite levels in adult zebrafish brain and head kidney tissue samples. It can be adapted for other fish species or other tissues without much hassle; however, tissue amounts may need to be adjusted.

Materials

MATERIALS

- Sodium nitrite **P212121**
- Disposable polystyrène cuvettes (1ml) **G-Biosciences Catalog #**786-009
- Griess Reagent System **Promega Catalog #**G2930
- 12×75mm test tubes **Fisher Scientific Catalog #**0555512
- Double-bean UV-visible spectrophotometer, with temperature control in the cuvette compartment
- PBS for zebrafish

STEP MATERIALS

- 12×75 mm high clarity polypropylene test tubes **Corning Catalog #**352063
- PBS for zebrafish
- Disposable polystyrène cuvettes (1ml) **G-Biosciences Catalog #**786-009
- Sodium nitrite **P212121**
- Distilled Water
- Sulfanilamide
- Chloridric acid 20%
- N-(1-Naphthyl)-ethylenediamine dihydrochloride **Bio Basic Inc. Catalog #**NB0650.SIZE.10g
- Chloridric acid 1%
- Centrifuge
- 12×75 mm high clarity polypropylene test tubes **Corning Catalog #**352063
- PBS for zebrafish
- Disposable polystyrène cuvettes (1ml) **G-Biosciences Catalog #**786-009
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- Sodium nitrite **P212121**
- Chloridric acid 20%
- Griess Reagent System **Promega Catalog #**G2930
- Sulfanilamide

Safety warnings

Make sure to read all Safety Data Sheets for the reagents. Sulfanilamide is hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, and of inhalation. NED causes skin irrtation, serious eye irritation, and may cause respiratory irritation. HCl is very hazardous in case of skin contact, of eye contact, and of ingestion. Therefore, use personal protective equipment whenever manipulating it. Moreover, reagents must be manipulated under a fume hood at all times.

Before start

Every biochemical protocol needs to be validated in the laboratory when first introduced. The present protocol describes validation steps that were taken in LaNeC.

Solutions

1 Nitrite stock solution:

1. Weight 0.015 g sodium nitrite ($NaNO_2$) and store in a beaker covered in aluminum foil
2. Dilute in 100 ml distilled water (volume measured in a volumetric flask)
3. Store in an ambar flask, identified and kept under refrigeration (2 - 8 ºC)

Sodium nitrite **P212121**

Distilled Water

Note

Nitrite solutions should be kept from light by storing in ambar flasks

Note

1 mL of this solution contains 14.4937 µM nitrite

2 Sulfanilamide stock solution:

1. Weight 0.5 g sulfanilamide and store in a beaker covered in aluminum foil
2. Dilute in 100 ml of HCl 20% (volume measured in a volumetric flask)
3. Store in an ambar flask, identified and kept under refrigeration (2 - 8 ºC)

Sulfanilamide

Chloridric acid 20%

Safety information

HCl is highly corrosive for skin
https://www.msdsonline.com/2014/09/10/hydrochloric-acid-hazards-safety-tips/

Note

Sulfanilamide solutions should be kept from light by storing in ambar flasks

3 NED [N-(1-Naphthyl)ethylenediamine] stock solution:

1. Weight 0.3 g NED and store in a beaker covered in aluminum foil
2. Dilute in 100 mL HCl 1% (volume measured in a volumetric flask)
3. Store in an ambar flask, identified and kept under refrigeration (2 - 8 ºC)

N-(1-Naphthyl)-ethylenediamine dihydrochloride **Bio Basic Inc. Catalog #**NB0650.SIZE.10g

Chloridric acid 1%

Assay validation: Linearity

4 Make serial dilutions of the $NaNO_2$ stock solution, adding volumes to aluminum foil-wrapped test tubes (Table on Step 7, below)

5 Add 0.5 mL sulfanilamide, suspend with vortex, and allow the solution to rest for 2 min.

6 Add 0.5 mL NED, suspend with vortex, and allow the solution to rest for 10 min.

7 Final volumes are as follows:

	Tube #	$NaNO_2$ vol (mL)	ddH_2O vol (mL)	Sulfanilamide vol (mL)	NED vol (mL)	Final volume	$[NO_2^-]$ (µM)
	1 (white)	0.0	24.0	0.5	0.5	25.0	0.0
	2	0.006	23.994	0.5	0.5	25.0	0.001769
	3	0.012	23.988	0.5	0.5	25.0	0.003538
	4	0.023	23.977	0.5	0.5	25.0	0.007077
	5	0.047	23.953	0.5	0.5	25.0	0.014154
	6	0.093	23.907	0.5	0.5	25.0	0.028308
	7	0.187	23.813	0.5	0.5	25.0	0.113232
	8	0.375	23.625	0.5	0.5	25.0	0.226464

12×75 mm high clarity polypropylene test tubes **Corning Catalog #**352063

8 Read the absorbance of each calibration solution with a spectrophotometer at 543 nm.

Note

ATTENTION
All analyses should be made with at least three technical replicates
Thoroughly rinse the cuvettes with distilled water to remove contaminants before each replicate

9 Data should be entered in two columns, 'x' and 'y', representing concentration and response, respectively. Plot data and perform a linear regression:

Software

R	NAME
Xubuntu 16.04	OS
https://www.r-project.org/	REPOSITORY

Command

Determine linearity and curve parameters using chemCal for R

```
if(!require(chemCal)){
   install.packages('chemCal')
   library(chemCal)
}
m <- lm(y ~ x, data = your_data)
summary(m)
calplot(m)
```

Software

chemCal for R	NAME
Xubuntu 16.04	OS
Johannes Ranke (https://orcid.org/0000-0003-4371-6538)	DEVELOPER
https://cran.r-project.org/web/packages/chemCal/index.html	REPOSITORY

10 Correlation coefficients higher than 0.98 suggest linearity. Error residuals should also be checked, looking for deviations in linearity, presence of atypical samples, heteroscedasticity, and dependence between errors; a well-adjusted curve should present erros with a uniform distribution, average zero and constant variance, and absence of atypical samples. An F-test comparing fits of residuals with a linear vs. quadratic model can also be used.

Assay validation: Sensitivity

11 The first step in determining the sensitivity of the assay is to determine the limit of detection (LOD). This can be done using parameters from the linear model, with the function 'lod' from the R package chemCal. Use alpha = 0.01 and beta = 0.5.

Software

chemCal for R	NAME
Xubuntu 16.04	OS
Johannes Ranke (https://orcid.org/0000-0003-4371-6538)	DEVELOPER
https://cran.r-project.org/web/packages/chemCal/index.html	REPOSITORY

Command

Calculate limit of detection using chemCal for R

```
lod(m, alpha = 0.01, beta = 0.5)
```

12 The next step in determining sensitivity of the assay is to determine the limit of quantification (LOQ). This can also be done using parameters from the linear model, with the 'loq' function from the package chemCal. Substitute 'your_n' by the number of technical replicates in the your assay.

Command

Determine limit of quantification with the chemCal package

```
loq(m, n = your_n)
```

Software	
chemCal for R	NAME
Xubuntu 16.04	OS
Johannes Ranke (https://orcid.org/0000-0003-4371-6538)	DEVELOPER
https://cran.r-project.org/web/packages/chemCal/index.html	REPOSITORY

Assay validation: Precision

13 Precision refers to the dispersion of measured values around an average value, and its numerical value is determined by the relative standard deviation (RSD):

RSD = 100 * s / $|\bar{x}|$
Where:
s = the sample standard deviation
$\bar{x}$ = sample mean

14 The first step in determining the precision is to assess the repeatability of the assay (that is, intra-run precision). Run three technical replicates for the lowest, intermediate, and highest NaO_2 concentrations. Determine intra-run precision by calculating the RSD for each of these concentrations. RSDs higher than 15% indicate low precision.

15 Intermediate precision, or inter-run precision, defines the precision of a measurement made in the same laboratory made by different analysts and/or different days. We follow ANVISA's recommendations by running three technical replicates for the lowest, intermediate, and highest NaO_2 concentrations in two different days and assessing the RSD for each concentration. RSDs higher than 15% indicate low intermediate precision.

Assay validation: Accuracy

16 Accuracy can be determined after the establishment of linearity, sensitivity, and precision. Run another calibration curve, with at least three technical replicates, for the entire range of concentrations. Accuracy is calculated as the difference between predicted (xi) and measured (x_v) absorbances

Predicted values can be obtained based on the linearity curve (Step 1) using a weighted linear model, with weights derived from the curve made for the accuracy determination. Using chemCal (where *new_data* refers to the curve made for accuracy determination)

Software

chemCal for R	NAME
Xubuntu 16.04	OS
Johannes Ranke (https://orcid.org/0000-0003-4371-6538)	DEVELOPER
https://cran.r-project.org/web/packages/chemCal/index.html	REPOSITORY

Command

Generate predicted values from weighted linear model (weights based on the results from the linearity assay)

```
weights <- with(new_data, { yx <- split(y, x) ybar <- sapply(yx,
mean) s <- round(sapply(yx, sd), digits = 2) w <- round(1 / (s^2),
digits = 3)
})
new_data.means <- aggregate(y ~ x, new_data, mean)
n <- lm(y ~ x, w = weights, data = new_data.means)
inverse.predict(new_data.means, 30, ws = your_weight) #your_weight
determined in previous step for 30 µM
inverse.predict(new_data.means, 15, ws = your_weight) #your_weight
determined in previous step for 15 µM
inverse.predict(new_data.means, 7.5, ws = your_weight) #your_weight
determined in previous step for 7.5 µM
inverse.predict(new_data.means, 3.75, ws = your_weight) #your_weight
determined in previous step for 3.75 µM
inverse.predict(new_data.means, 1.875, ws = your_weight) #your_weight
determined in previous step for 1.875 µM
inverse.predict(new_data.means, 0.9375, ws = your_weight)
#your_weight determined in previous step for 0.9375 µM
inverse.predict(new_data.means, 0.46875, ws = your_weight)
#your_weight determined in previous step for 0.46875 µM
inverse.predict(new_data.means, 0.234375, ws = your_weight)
#your_weight determined in previous step for 0.234375 µM
```

17 Use the predicted values x_i obtained in the previous step to calculate the accuracy at each level, as:

Accuracy = ([xi - x_v] / x_v) * 100

Biological sample preparation

18 Sacrifice animal with ice-cold water (< 12 ºC) followed by spinal transection.

19 Carefully dissect the brain (http://dx.doi.org/10.3791/1717) and head kidney (http://dx.doi.org/10.3791/2839).
http://dx.doi.org/10.3791/1717 http://dx.doi.org/10.3791/2839

20 Homogenize the tissue samples in 500 µL Zebrafish PBS (0.1 M, pH 7.3). Due to the low sensitivity of the assay, tissues need to be homogenized in a pool from 5 animals.

Note

Tissues can be kept frozen (- 20 ºC) for up to 6 months.

Note

Due to the low sensitivity of the assay, tissues need to be homogenized in a pool from 5 animals.

PBS for zebrafish

21 Centrifuge samples for 10 min at 14,500 rpm. The supernatant is used for the assay.
Centrifuge

Measure nitrite levels

22 Run a calibration curve by following the steps for linearity (Steps 4-8)

23 Re-suspend the supernatant by vortexing, if needed.

24 Carefully transfer 250 µL of the sample to the cuvette.
Disposable polystyrène cuvettes (1ml) **G-Biosciences Catalog #**786-009

25 Add 125 µL sulfanilamide solution, vortex, and let it rest for 2 min

26 Add 125 µL NED solution, vortex, and let it rest for 10 min

27 Measure absorbance at the spectrophotometer at 542 nm.

28 Determine NO_2^- levels in the sample by interpolating from a calibration curve (Step 22). Inverse prediction can be made using chemCal for R

Software

chemCal for R	NAME
Xubuntu 16.04	OS
Johannes Ranke (https://orcid.org/0000-0003-4371-6538)	DEVELOPER
https://cran.r-project.org/web/packages/chemCal/index.html	REPOSITORY

Command

Interpolate unknowns (sample nitrite) from the calibration curve
m refers to the values derived from the linear model, and unknown_value to the absorbances measured in the assay

```
inverse.predict(m, unknown_value, alpha = 0.01)
```

29 Correct nitrite values by protein levels, measured using the Bradford assay.