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

Caio Maximino,Jeisiane Souza de Oliveira,Monica Gomes Lima-Maximino

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.

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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.

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.

Materials

Sodium nitrite View by P212121

Disposable polystyrène cuvettes (1ml) 786-009 by G-Biosciences

Griess Reagent System G2930 by Promega

12x75mm test tubes 0555512 by Fisher Scientific

Double-bean UV-visible spectrophotometer, with temperature control in the cuvette compartment by Contributed by users

PBS for zebrafish View by Contributed by users

Protocol

Solutions

Step 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)

REAGENTS

Sodium nitrite View by P212121

Distilled Water by Contributed by users

NOTES

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

1 mL of this solution contains 14.4937 µM nitrite

Solutions

Step 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)

REAGENTS

Sulfanilamide by Contributed by users

Chloridric acid 20% by Contributed by users

SAFETY INFORMATION

HCl is highly corrosive for skin

NOTES

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

Solutions

Step 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)

REAGENTS

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

Chloridric acid 1% by Contributed by users

Assay validation: Linearity

Step 4.

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

Assay validation: Linearity

Step 5.

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

Assay validation: Linearity

Step 6.

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

Assay validation: Linearity

Step 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

REAGENTS

12x75 mm high clarity polypropylene test tubes 352063 by Corning

Assay validation: Linearity

Step 8.

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

NOTES

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

Assay validation: Linearity

Step 9.

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

SOFTWARE PACKAGE (Xubuntu -)

chemCal for R, 0.2.1

Johannes Ranke (https://orcid.org/0000-0003-4371-6538)
https://cran.r-project.org/web/packages/chemCal/index.html

cmd COMMAND

```
if(!require(chemCal)){
   install.packages('chemCal')
   library(chemCal)
}

m <- lm(y ~ x, data = your_data)

summary(m)

calplot(m)
```

Determine linearity and curve parameters using chemCal for R

Assay validation: Linearity

Step 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

Step 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 PACKAGE (Xubuntu -)

chemCal for R, 0.2.1

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https://cran.r-project.org/web/packages/chemCal/index.html

cmd COMMAND

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

Calculate limit of detection using chemCal for R

Assay validation: Sensitivity

Step 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.

SOFTWARE PACKAGE (Xubuntu -)

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https://cran.r-project.org/web/packages/chemCal/index.html

cmd COMMAND

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

Determine limit of quantification with the chemCal package

Assay validation: Precision

Step 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

Assay validation: Precision

Step 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.

Assay validation: Precision

Step 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

Step 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)

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cmd COMMAND

```
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 s
tep for 30 µM
inverse.predict(new_data.means, 15, ws = your_weight) #your_weight determined in previous s
tep 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 previou
s step for 1.875 µM
inverse.predict(new_data.means, 0.9375, ws = your_weight) #your_weight determined in previo
us step for 0.9375 µM
inverse.predict(new_data.means, 0.46875, ws = your_weight) #your_weight determined in previ
ous step for 0.46875 µM
inverse.predict(new_data.means, 0.234375, ws = your_weight) #your_weight determined in prev
ious step for 0.234375 µM
```

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

Assay validation: Accuracy

Step 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

Step 18.

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

Biological sample preparation

Step 19.

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

LINK:
http://dx.doi.org/10.3791/2839

Biological sample preparation

Step 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.

REAGENTS

- PBS for zebrafish View by Contributed by users

NOTES

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

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

Biological sample preparation

Step 21.

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

REAGENTS

- Centrifuge by Contributed by users

Measure nitrite levels

Step 22.

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

Measure nitrite levels

Step 23.

Re-suspend the supernatant by vortexing, if needed.

Measure nitrite levels

Step 24.

Carefully transfer 250 µL of the sample to the cuvette.

REAGENTS

- Disposable polystyrène cuvettes (1ml) 786-009 by G-Biosciences

Measure nitrite levels

Step 25.

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

Measure nitrite levels

Step 26.

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

Measure nitrite levels

Step 27.

Measure absorbance at the spectrophotometer at 542 nm.

Measure nitrite levels

Step 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

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https://cran.r-project.org/web/packages/chemCal/index.html

cmd COMMAND

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

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

Measure nitrite levels

Step 29.

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

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.