NITRITE/NITRATE ASSAY
9/20/95

Equipment:
Molecular Devices Spectromax plate reader
micro pipettors for accurate delivery and mixing of small volumes in a 96 well plate format.

Solutions:

Nitrite Standard
Frozen stock prepared from dissolving 69mg sodium nitrite in l000cc dist. H_20. (1mM $N0_2$)
To prepare std. curve, thaw out 1 vial of l mM N02, dilute 1/10 in PBS or specific media for 100 uM std. Prepare std curve from 100 uM $N0_2$ std.
use 0,1,5, 10, 15,20,30,40,50 uM for standard curve

Nitrate Standard
Frozen stock prepared from dissolving 84mg sodium nitrate in l000cc dist. H_20. (l mM $N0_3$)
To prepare std. curve, thaw out 1 vial of lmM $N0_3$, dilute 1/10 in PBS or specific media for 100 uM std. Prepare std. curve from 100 uM $N0_3$ std.
use 0,1,5,10,15,20,30,40,50 uM for standard curve

Tris Buffer—40mM Tris pH 7.6

Nitrate Reductase (prepared fresh and kept on ice) 10 enzyme unit vials--dissolve contents in 16.4cc 40mM Tris pH 7.6

NADPH2 (prepared fresh and kept on ice)
6.7mg NADPH2 dissolved in 50cc of 40mM Tris pH 7.6

Griess 1 sol'n
1% Sulfanilamide in 5% phosphoric acid

Griess 2 sol'n
0.1% naphthyl-ethylenediamine in dist. H_20

Griess working so
mix griess 1 and 2 1:1 ratio prior to use

Assay:
The assay is done in two parts, nitrate reductase is reacted with the sample, and then the Griess assay is used for nitrite. If nitrite alone is needed, then omit the nitrate reductase
The assay can be set-up in test tubes or 96-well plates. The Griess method is set-up in 96—well plate format and read on a Spectrornax plate reader.

FOR NITRATE REDUCTASE REACTION:
1. add 50u1 water blank, media blank, standard, or sample to well
2. add 25u1 nitrate reductase sol'n
3. add 25u1 NADPH sol'n
4. incubate (room temp.) 2hrs for tissue culture, 3hrs for plasma
FOR GRIESS ASSAY:
5. add 100 ul working griess sol'n
6. incubate 10 min
7. read on plate reader at 550nN, correcting for abs. at 650 nM

Note: 1.plasma samples may have to be diluted 1/10 with PBS as we as running a PBS blank to correct for background absorbance.
2. std. curve should be prepared in PBS when running plasma, and prepared in the specific cell media used for experiments when culture media is analyzed.
3. the nitrate reductase and NADPH, once prepared in tris buffer, can be re-frozen and stored at -20 C for future assays.
4. The nitrate reductase vials we order have 10 enzyme units per vial. The actual mg content may vary. Use 16.4ml tris buffer for each 10 units of enzyme.

Protocol a Netről

Greiss Assay protocol

Solutions:

2% sulphanilamide, 5% H_3PO_4 (phosphoric acid)

0.2% NAP (napthylethylenediamine)

Standards:

Sodium Nitrite (@ 6.9 mg/ml) = 100 mM

Standard Dilutions (to produce calibration curve):

- S1= 1:1000 dilution of sodium nitrate stock (100 uM)
- S2= 500 ml tissue culture media + 500 ml S1 (50 uM)
- S3= 500 ml media + 500 ml S2 (25 uM)
- S4= 500 ml media + 500 ml S3 (12.5 uM)
- S5= 500 ml media + 500 ml S4 (6.25 uM)
- S6= 500 ml media + 500 ml S5 (3.125 uM)
- S7= 500 ml media + 500 ml S6 (1.56 uM)
- S8= 500 ml media + 500 ml S7 (0.78 uM)

Blank = 75 ul tissue culture media

Protocol:

Mix solution 1 and 2 in a 1:1 ratio (1 ml of each, solution should be colourless). Add 25 ul of this to 75 ul of sample, standard or blank. Measure at 540 nm.

This protocol is suitable for the detection of nitrite and can be related to the production of NO. However, NO can form both nitrite and nitrate in varying proportions. For some purposes it is possible to simply measure nitrite and to compare the levels of nitrite between samples to estimate the level of NO produced. However, in many cases it is necessary to determine the total amount of NO produced by measuring both nitrate and nitrite. To do this the nitrate must be first converted to nitrite and then the nitrite levels measured with the assay described above.

Nitrate can be converted to nitrite by using the enzyme nitrate reductase (NR) from Aspergillus. The protocol for this is as follows:

- Mix 300 ul of media or cell lysate with 10 ul of nitrate reductase solution (0.1 U/ml nitrate reductase enzyme, 5 mM FAD, 30 mM NADPH).
- Incubate at 37^{o}C for around 15 minutes
- NADPH now needs to be oxidized as it will otherwise interfere with the Greiss reaction. To achieve this add 10 ul of lactate dehydrogenase solution(100 U/ml rabbit muscle lactate dehydrogenase enzyme and 0.3 mM sodium pyruvate).
- Incubate at 37^{o}C for around 5 minutes, before proceeding to the Greiss assay.

Analysis of NO_2
$_2$ 1 NO_3
$_2$ required reduction of NO_3
$_2$ to
NO_2
$_2$ with aspergillus nitrate reductase (Sigma Chemical). All samples were run in duplicate. Nitrate reductase 50 ml (0.2 U), FAD 5 ml (5 mmol/liter), NADPH 5 ml (6 mmol/liter) and phosphate buffer (1.2 mmol/liter) were added to 50 ml of deproteinized plasma to yield a final volume of 150 ml and subsequently incubated at 36°C for 1 h to allow for sufficient conversion of NO_3
$_2$ to NO_2
$_2$.